A DISSERTATION FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN HUMAN ECOLOGY

Construction of Recombinant *Bifidobacterium bifidum* BGN4 Expressing Bifidobacterial β-Galactosidase Gene and Application for Improvement of Lactose Intolerance

Bifidobacterium 유래 β-Galactosidase 유전자를 발현하는 *B. bifidum* BGN4 재조합체의 구축과 유당불내증 개선에의 적용

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Abstract

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The aim of this study was to obtain a recombinant bifidobacteria with high β-galactosidase activity through molecular genetic techniques and apply it *in vitro* lactose hydrolysis and *in vivo* lactose-intolerance animal experiments. Incidentally, development of a selection marker for a food-grade vector for bifidobacteria was pursued to enable recombinant bifidobacteria to be used in various applications for the human body and food product in the future.

Prior to the development of the β-galactosidase over-expressing bifidobacteria, a preliminary study to improve the electroporation efficiency of bifidobacteria was performed. As a result, the electroporation efficiency of *B. bifidum* BGN4, which was a transformation host in my study, was drastically and consistently increased from 10^3 to 10^5 CFU / μg DNA. The optimized electrotransformation conditions were widely applicable to other *Bifidobacterium* species.

Returning to the main study, to obtain β-galactosidase over-expressing bifidobacteria, the β-galactosidase gene (G1) was cloned from *Bifidobacterium longum* RD47 (RD47), which is one of the bifidobacterial strains with the highest level of β-galactosidase activity, with combinations of several bifidobacterial promoters and expressed in *B. bifidum* BGN4 (BGN4). Among the recombinant bifidobacteria, BGN4-G1 exhibited the highest β-galactosidase level, for which the hydrolytic activity was continuously 2.5 to 4.2 times higher than that of BGN4 and 4.3 to 9.6 times higher than that of RD47. The β-galactosidase activity of BGN4-G1 was exceedingly superior to that of any of the other 35 experimental lactic acid bacteria. BGN4-G1 could remove 50% of the lactose from the whole milk already at 63 h and finally 61% at 93 h. This figure is about twice the lactose removal rate of conventional fermented milk. The crude enzyme extract containing 50 μg of protein from BGN4-G1 could remove 51% of the lactose in milk within 2 h. Therefore, it

is believed that the purification of G1 from BGN4 by attaching a his-tag to the gene will have industrial significance, such as using it in the production of lactose-free milk or fermented milk.

Lactose intolerance (LI) is a worldwide issue in terms of public health management. Recently, *Bifidobacterium* species has attracted attention as a potential treatment for lactase deficiency. However, contradictory results have been reported in previous studies in which bifidobacteria were effective for LI or not. I hypothesized that bifidobacteria may be effective in alleviating LI, but the reason for the contradictory results in the previous studies were due to differences in the β-galactosidase activities among *Bifidobacterium* strains used in the experiments. To prove the hypothesis, it was tried to verify the effect of β-galactosidase activities of bifidobacteria on LI alleviation by feeding post-weaning Balb/c mice with various version of *Bifidobacterium* strains with different β-galactosidase activities (β-galactosidase reduced mutant, wild-type and β-galactosidase over-expressing bifidobacteria). As a result, the LI related symptoms such as total number and total weight of feces and intestinal motility have been improved according to the increased activity of β-galactosidase of bifidobacteria. The LI alleviation effect in the BGN4-G1 administration group was greater than that in the lactase-treated group and even reached a LI-uninduced level. Meanwhile, the stool frequency and total feces weight of the BGN4 and BGN4-bgr administration groups tended to decrease compared

to the untreated group, but no significant difference was found. In the case of intestinal motility, in contrast to the BGN4-bgr group, the BGN4 administration group was significantly inhibited in intestinal motility compared to the untreated group. This suggests that the LI alleviation effect is improved as the β-galactosidase activity of the bifidobacteria is increased.

To explore the mechanisms for improved LI according to the β-galactosidase activity, a gut microbiome analysis was performed from the feces of the mice fed BGN4-bgr, BGN4, BGN4-G1 or NS for two weeks. As a result, some meaningful results were derived as follows: 1) The proportion of several microorganisms associated with intestinal health tended to be differentiated between the *Bifidobacterium*-fed groups and the untreated group, 2) The bifidobacterial proportion in the BGN4-G1 group tended to be increased compared to the other *Bifidobacterium*-fed groups, 3) The balance of SCFAs and lactate was differentiated between the *Bifidobacterium*-fed groups and the untreated group, 4) The rates for the change in SCFAs and lactate were grouped in a similar pattern in the BGN4-G1 and BGN4 groups except for the BGN4-bgr group.

Because the BGN4-G1 exhibited a significant effect on LI alleviation, it is expected that BGN4-G1 can be reproduced with a food-grade vector for bifidobacteria, and it will be used as an LI treatment strain. However, food-grade vectors for bifidobacteria have not been developed yet to my knowledge, so it was decided to develop a

selection marker for constructing a food-grade vector for bifidobacteria using the superoxide dismutase (SOD) and catalase genes from lactic acid bacteria as food grade selection marker. First, the SOD and catalase genes were subcloned into pBES2 with combinations of bifidobacterial promoters and a terminator and transformed into BGN4 to construct BGN4-SK. For the efficient selection of transformant using H_2O_2, the optimal H_2O_2 concentration and exposure time were investigated. As a result, when a bacterial mixture of 10^2 CFU of BGN4-SK and 10^8 CFU of wild-type BGN4 was treated with 4 mM H_2O_2 stress for 2 h, most of the cells were dead and only 34 colonies remained, of which 2 colonies were identified as BGN4-SK. It suggests that the selection pressure induced by H_2O_2 is suitable for the selection of a few recombinants BGN4 among the majority of wild-type BGN4. It is meaningful in that the SOD and catalase genes can be a candidate food grade selection marker for bifidobacteria.

Keywords : β-galactosidase, recombinant bifidobacteria, *Bifidobacterium longum* subsp. *longum* RD47, *Bifidobacterium bifidum* BGN4, electroporation-mediated transformation efficiency, lactose hydrolysis, lactose intolerance, food-grade vector

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Chapter 1.

Literature review

1.1 Properties and structure of the β-galactosidase

β-galactosidase (EC 3.2.1.23) also known as lactase catalyzes the hydrolysis of the terminal β-1, 4-D-galactosyl residues found in β-D-galactosides, such as lactose, and releases D-galactose and D-glucose as a final product (Kamran et al., 2016; Nguyen et al., 2015; Yuan et al., 2008). In addition to hydrolysis, some of the β-galactosidases catalyze the reverse reaction of the hydrolysis called transgalactosylation (Maksimainen et al., 2011). β-galactosidases are abundant in animals, plants and microorganisms. These enzymes are classified as family 2 (GH2) and 35 (GH35). While approximately 70% of those derived from GH35 are found in plants, those derived from GH2 are found primarily in microorganisms (De Alcantara et al., 2006). Because of their ability for lactose hydrolysis and transgalactosylation, β-galactosidase has several main technological applications in the food industry: development of lactose-removed milk and dairy products, production of galacto-oligosaccharides during lactose hydrolysis and use as a component of tablet-type β-galactosidase products for alleviation of lactose intolerant symptoms (Li et al., 2012; Nguyen et al., 2012). For these industrial purposes, β-galactosidases are mainly extracted from

microbes including *Bifidobacterium* (Hsu et al., 2007; Møller et al., 2001) and fungi. The production of β-galactosidase from microorganisms is a preferred choice because of the relatively low cost of the enzyme and a higher yield. By extension, to safely use β-galactosidase in the food industry, the microorganisms producing β-galactosidase must be food-grade. In this respect, *Bifidobacterium* is considered to be a good source of β-galactosidase due to their GRAS (Generally recognized as safe) status (Chanalia et al., 2018).

Although the structure of β-galactosidase varies depending on the source of the enzyme (Rosenberg, 2006), β-galactosidase is typically a tetramer of four identical polypeptide chains. Within each monomer, the constituent amino acids form five well-defined structural domains (Juers et al., 2012). One of these five domains forms a jelly roll barrel, and the remaining domains are composed of fibronectin, β-sandwich and TIM (triose phosphate isomerase)-type barrel which is the structure of the central domain (Saqib et al., 2017). In the central domain, there is an active site that forms a deep pocket at the C-terminal end of the TIM-type barrel (Juers et al., 2012). All residues identified by biochemical experiments as being important for catalysis are located in or near this pocket. Monovalent and divalent cation binding sites including a sodium/potassium site and a magnesium site have been identified in the active site (Juers et

al., 2001). The activity of the active site is controlled by the dissociation of the tetramer into the dimer (Saqib et al., 2017). The structure of β-galactosidase is illustrated in Fig. 1.1.

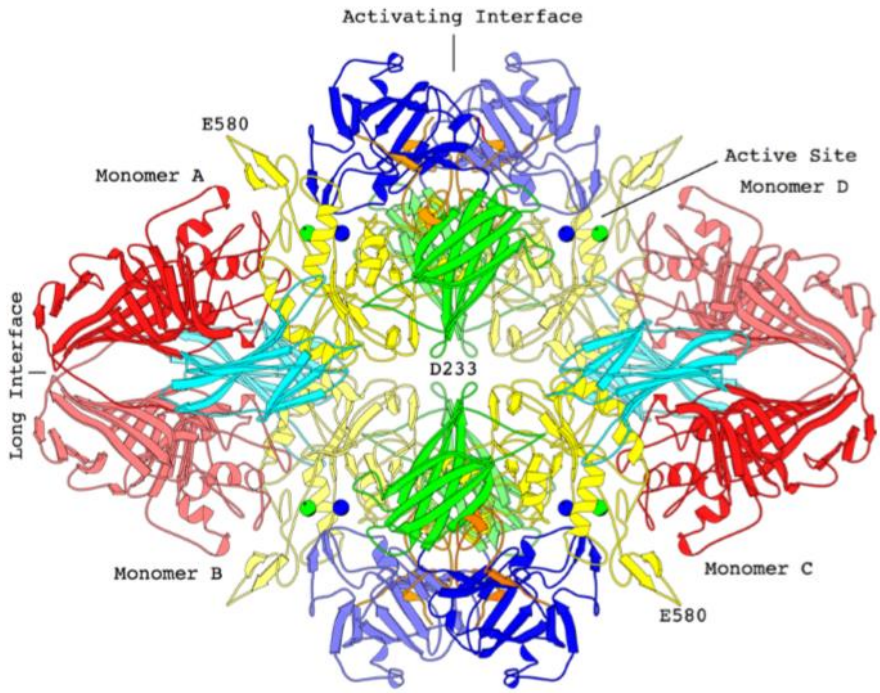


Fig. 1.1 The tetrameric structure of β−galactosidase

Domain 1, blue; Domain 2, green; Domain 3, yellow; Domain 4, cyan; Domain 5, red; Na^{+}, green sphere; Mg^{2+}, blue sphere. Lighter and darker shading are used to distinguish between equivalent domains in different subunits.

Cited from (Juers et al., 2012).

1.2 The role of β-galactosidase in the food industry

1.2.1 Removal of lactose from milk

β-galactosidase has a crucial role in the food industry, especially in the dairy industry. Complete removal of lactose in milk using β-galactosidase is necessary to address the problem of lactose intolerance that occurs in about 70% of the world's population. Furthermore, this reaction has other benefits in food industry such as prevention of lactose crystallization and the increase of sweetness (Benavente et al., 2015). There are 2 methods for removing lactose from milk: adding the enzyme to milk to make lactose-free milk and reacting the milk with lactic acid bacteria to make fermented milk products.

Currently, there are two processes (batch and aseptic) to produce lactose-free milk (Harju, 2004; Troise et al., 2016) and β-galactosidase is used in both processes. A schematic representation of the batch and aseptic processes is illustrated in Fig. 1.2, and the figure shows the time point at which the enzyme is added in each process. The enzymatic hydrolysis of lactose in milk has two alternatives: use of immobilized or soluble β-galactosidase. In the past, immobilization of β-galactosidase had attracted much

attention as a promising method of reducing the cost of the lactose hydrolysis due to repeated use of the enzyme for industrial processes (Rosenberg, 2006). However, immobilization of β-galactosidase has not been used in industrial practice for the production of lactose-free milk to date, due to issues with the microbial stability of the final product (Dekker et al., 2019). For this reason, only soluble β-galactosidase is currently used in the batch and aseptic processes for lactose-free milk production. In the batch process, β-galactosidase is added to milk that is not sterilized yet. This process should be performed under cooled conditions (typically 4 - 8℃) to prevent microbial growth. After the reaction with the enzyme, the milk is pasteurized, homogenized and packaged. Because of the cooled conditions and the limited incubation time, the batch process requires a large amount of β-galactosidase, and the enzymes available in this process must have a relatively high hydrolytic activity at a neutral pH and low temperature. When the lactose-free milk is produced through the batch process, there is an advantage that no residual β-galactosidase activity remains in the final product because the enzyme is inactivated during the pasteurization of the milk (Dekker et al., 2019). Meanwhile, in the aseptic process, filter-sterilized β-galactosidase is added to UHT (ultra-heat treated)-sterilized milk just before packaging (Dahlqvist et al., 1977). The packaged milk is stored under moderate temperature for approximately 3 days before shipping, during which lactose hydrolysis occurs. Because the

incubation time and temperature for the aseptic process are higher than for the batch process, the aseptic process requires a relatively low amount of β-galactosidase. However, there is a weakness that process control can not be achieved because the enzyme is only active in the milk package; thus, the highest quality β-galactosidase should be used for the aseptic process to prevent problems during storage (Dekker et al., 2019).

The lactose content in milk can also be reduced by the fermentation of lactic acid bacteria, such as *Lactobacillus*, *Lactococcus* and *Leuconostoc,* which have their own β-galactosidase activity. Lactic acid bacteria cause the acidification of milk by the production of organic acids, mainly lactate. Furthermore, they produce acetate, aroma compounds, exopolysaccharides, bacteriocins and several enzymes during the fermentation, which enhance the microbial safety and shelf life, and improve the texture and sensory profile of the final product (Leroy and De Vuyst, 2004). Most fermented milk products still contain a significant amount of lactose because lactic acid bacteria can only remove about 30% of the lactose in milk on average, which can cause problems for lactose-intolerant people when consuming these dairy products (Harju et al., 2012). To overcome these shortcomings, β-galactosidase is sometimes added together with lactic acid bacteria when making yogurt in the food industry. Because the pH of the milk fermented by the lactic acid bacteria drops below 5.5 within several hours, a large amount of the enzyme is

needed when the neutral β-galactosidase is used because the enzyme is rapidly inactivated under acidic condition. In the case of using the acid stable enzyme, the amount of enzyme required is relatively less, but there is a problem in that the enzymes are not inactivated in the final product (Dekker et al., 2019).

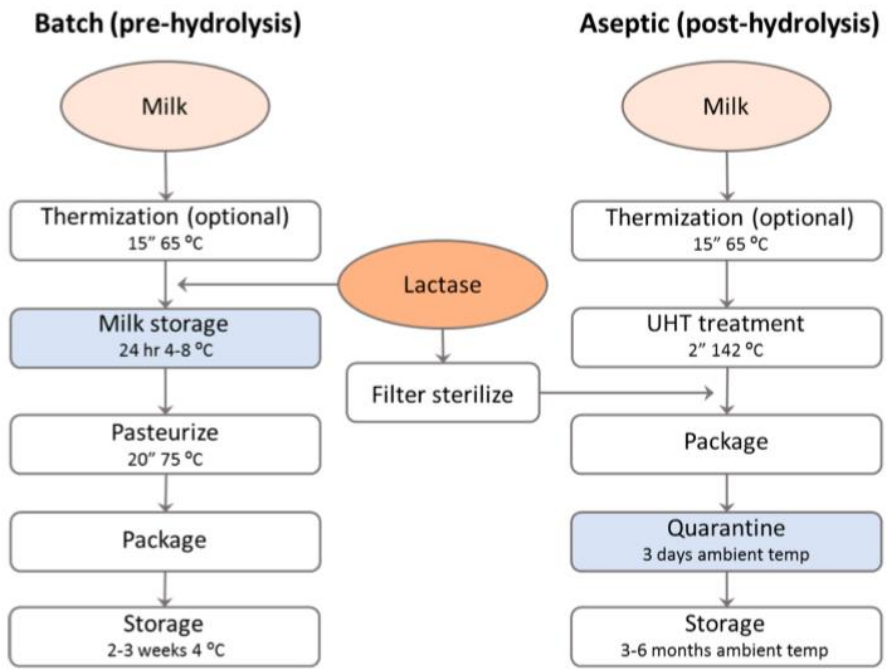


Fig. 1.2 A schematic representation of the batch and aseptic processes

Cited from (Dekker et al., 2019)

1.2.2 Production of galacto-oligosaccharides

Galacto-oligosaccharides (GOS) are produced by the transgalactosylation activity of β-galactosidase during the hydrolysis of lactose (Saqib et al., 2017). A schematic process of transgalactosylation by β-galactosidase is illustrated in Fig. 1.3. The conversion of lactose to GOS by β-galactosidase is regulated by competition between hydrolysis and transgalactosylation. Transgalactosylation involves intramolecular and intermolecular reactions. Intramolecular galactosyl transfer to D-glucose yields regio-isomers of lactose and intermolecular galactosyl transfer produces GOS with various degrees of polymerization (DP) from lactose (Torres et al., 2010). In general, the yield of the GOS synthesis can be improved as follows: using micro-aqueous conditions; using a highly concentrated lactose solution; modifying the enzyme; and removing the final product or inhibitors from the reaction mixture (Czermak et al., 2004; Monsan and Paul, 1995). Currently, a number of studies have focused on GOS production from microorganism-derived β-galactosidases. Because GOS has been reported to specifically promote the growth of the *Lactobacillus* and *Bifidobacterium* species and reduce the number of pathogenic bacteria in the human intestine, they are used as prebiotics in the food industry (Møller et al., 2001). Nowadays, GOS is used extensively in a variety of applications such as alternative

sweeteners, cosmetics, soft drinks, infant food, cereals, and powdered milk (Nagy et al., 2001).

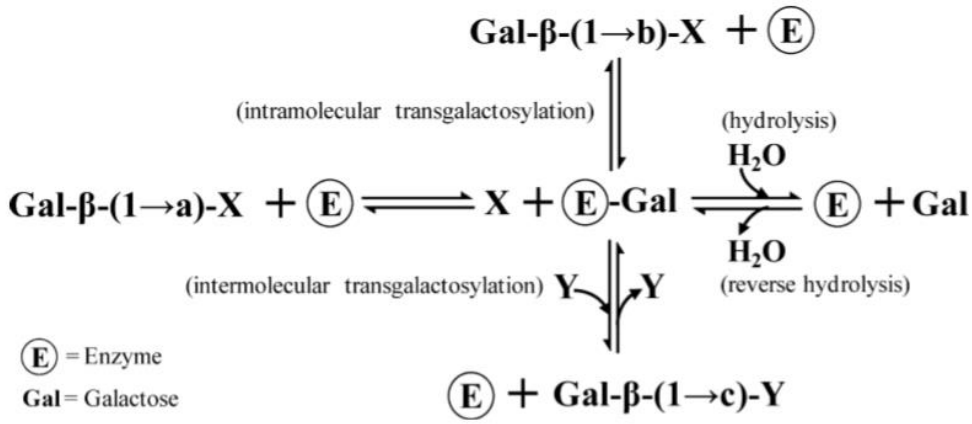


Fig. 1.3 General process of transgalactosylation by β-galactosidase

a, b, and c: 2, 3, 4, 6 (a≠b), indicate the glycosidic linkage position. X, galactosyl donor; Y, galactosyl acceptor.

Cited from (Torres et al., 2010).

1.3 The role of β-galactosidase (lactase) in lactose intolerance alleviation

Lactose intolerance (LI) is a clinical syndrome characterized by the inability to digest lactose due to a deficiency in lactase activity in the intestine. Lactase is localized to the brush border of the small intestinal enterocytes. It hydrolyzes dietary lactose into glucose and galactose for proper absorption. If lactase in the small intestine is deficient or absent, unabsorbed lactose in the small intestine is passed to the large intestine and fermented by the colonic microflora. During fermentation, lactose is converted into gas and short chain fatty acids, and lactose itself and the fermentation products of lactose osmotically attract fluid into the bowel (Vonk et al., 2012). After the consumption of lactose-containing food, the following typical symptoms appear in lactose-intolerant people: abdominal pain, nausea, diarrhea, flatulence and bloating. The LI symptoms vary from individual to individual, depending on the amount of lactose ingested, the degree of lactase deficiency, and the form of lactose-containing food (Heyman, 2006).

Lactase deficiency occurs as three main types: primary, secondary, and congenital. Primary lactase deficiency is the most common cause of LI, accounting for about 70% of the world population. It is caused by a gradual decrease of lactase activity in the small intestine with

age (Heyman, 2006). Secondary lactase deficiency results from a reduced level of lactase in the small bowel due to malnutrition, cancer, diarrhea, inflammatory bowel disease, or other causes of injury to the small intestine. It can occur at any age and is a transient issue. Congenital lactase deficiency is the most severe and extremely rare. A patient with congenital lactase deficiency has a completely reduced or deficient lactase activity in the small intestine at the genetic level (Nivetha and Mohanasrinivasan, 2017).

The fundamental treatment for LI is to exclude milk from the diet of LI people. However, this approach can lead to severe nutritional deficiencies in calcium, phosphorus and vitamins (Montalto et al., 2006) and may reduce the quality of life due to dietary restrictions. As an alternative to this, fermented milk is generally regarded as the ideal milk substitute for LI subjects. However, consuming fermented milk instead of milk also has a limitation because only about 30% of the lactose is reported to be removed from fermented milk products (Harju et al., 2012). To overcome the limitations of the dietary approach, alternative strategies are emerging, such as the use of exogenous β-galactosidase and probiotics for their bacterial β-galactosidase activity (Montalto et al., 2006).

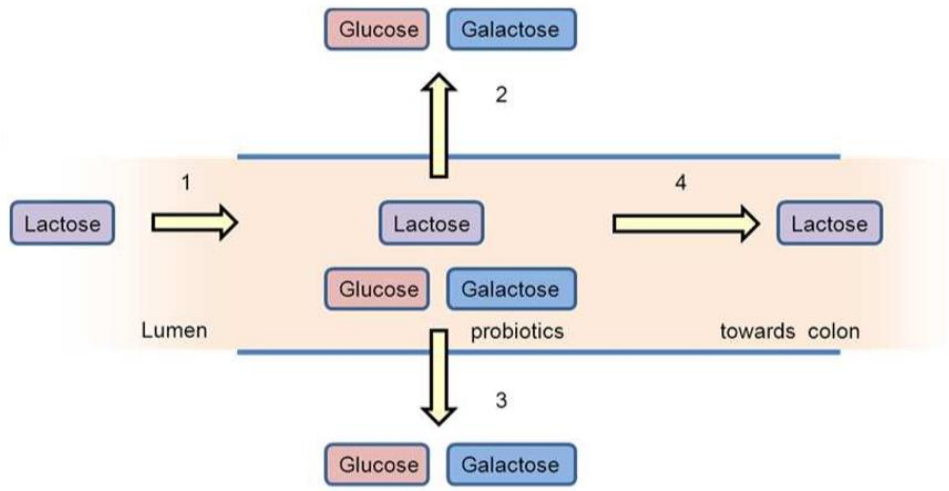


Fig. 1.4 Small intestinal metabolism of lactose

Lactose entering the small intestine (1) is converted to glucose and galactose by lactase (2) or probiotics (3). Excess amount of lactose is passed to the large intestine (4).

Cited from (Vonk et al., 2012).

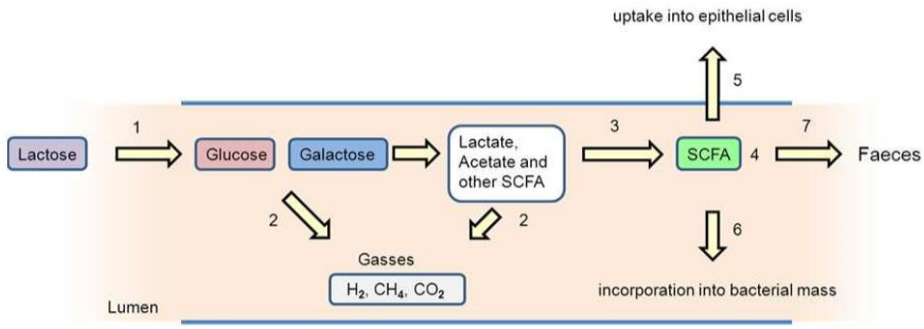


Fig. 1.5 Colonic metabolism of lactose

Lactose entering the colon (1) is fermented by the gut microbiota. During the fermentation, gasses are generated by the gas-producing bacteria (2) and lactate and SCFAs (short chain fatty acids) are produced as a fermentation product (3, 4). SCFAs can be absorbed by the epithelial cells (5), used by the gut microbiota (6) or excreted into the feces (7).

Cited from (Vonk et al., 2012).

1.3.1 Exogenous β-galactosidase

β-galactosidase has been marketed in a tablet form for LI subjects. These tablets contain β-galactosidase active at a low pH, which enables the enzyme to function in the stomach. It is recommended that the tablet should be taken immediately before the consumption of lactose-containing food. Previous studies have reported that exogenous enzyme alleviates LI symptoms (Medow et al., 1990; Xenos et al., 1998). However, taking tablets of exogenous β-galactosidase is inconvenient because it should be consumed whenever eating lactose-containing food and is not very effective due to rapid degradation of the β-galactosidase activity (Li et al., 2012).

1.3.2 Endogenous β-galactosidase expressed in probiotics

Probiotics, in particular the *Bifidobacterium* and *Lactobacillus* species, have received attention as a potential compensation for lactase deficiency, and many related clinical trials have been reported (Oak and Jha, 2018). The mechanisms by which probiotics exert their LI alleviation effect have not been fully revealed but may involve expressing their β-galactosidase activity, modifying the intestinal pH and positively affecting bowel functions and the gut microbiota (He et al., 2008).

The effects of probiotics on LI can be expected at two levels: removal of lactose in lactose-containing foods and in the small intestine, and during fermentation in the large intestine. Live probiotics in fermented milk products such as yogurt remove lactose in the product, and these lactose-reduced products are ingested as a milk substitute for LI subjects. Probiotics are usually consumed in the form of bacterial powder or in the form contained in fermented products. Ingested probiotics can increase the overall lactose hydrolysis of the small intestine. The β-galactosidase activity of probiotics is usually optimal between pH 6 – 8 in the small intestine. However, in the colon, where the pH decreases to 4, bacterial β-galactosidase activity is limited, and the lactose is more likely to remain unfermented. In this case, osmotic pressure is increased by

the residual lactose, and symptoms of lactose intolerance are caused by this increased osmotic load. The variable ability of the gut microbiota to ferment lactose in LI subjects may explain why different subjects have different levels of LI symptom (Lomer et al., 2008). In reality, several studies have shown that the balance of the intestinal microflora affects LI alleviation (Kopp-Hoolihan, 2001; Masood et al., 2011).

It has been reported that regular intake of probiotics can reduce LI symptom. He T, *et al.* (He et al., 2008) showed that *Bifidobacterium longum* capsules in yogurt increase the β-galactosidase activity in feces and alleviate LI. A mixture of *Lactobacillus casei* and *Bifidobacterium breve* also improves LI symptoms (Almeida et al., 2012) and yogurt containing *Lactobacillus acidophilus* relieved LI (Fuller, 1991). Other probiotic strains also have shown advantageous effects on lactase deficiency (Hiele et al., 1988; Lin et al., 1993; Rabot et al., 2010). However, contradictory results have also been reported that probiotics do not alleviate LI. Saltzman JR, *et al.* (Saltzman et al., 1999) observed that *Lactobacillus acidophilus* consumption did not change the breath hydrogen level or LI symptoms and Park MJ, *et al.* (Park et al., 1999) showed *Bifidobacterium breve* supplementation did not alleviate LI. With respect to these conflicting results, Kara M. Levri *et al. (Levri et al., 2005)* suggested that specific probiotic strains seem to be beneficial for lactase deficiency; thus, further researches on specific strains are

necessary to elucidate this potential therapeutic relationship. Identifying the LI alleviation effects of specific probiotic strains and their mechanisms of action through follow-up studies will be of great help in dealing with LI which is regarded as a worldwide issue.

TABLE 2

Does the addition of probiotics to non-fermented dairy products decrease lactose intolerance at a single meal?

LEAD AUTHOR	BREATH HYDROGEN RESULTS*	SYMPTOM RESULTS	COMMENTS
Dehkordi (1995)	Negative (1 of 1 Expt 1; 2 of 2 Expt 2)	Not measured	*Non-probiotic* treatment arm of whole milk with corn flakes "alleviated lactose malabsorption significantly" ($P<.01$).[11]
Jiang (1996)	Positive (2 of 3)	Negative (2 of 3 flatulence, 3 of 3 abdom. pain, 3 of 3 meteorism, 3 of 3 borborygmi, 3 of 3 diarrhea)	
Mustapha (1997)	Positive (3 of 4)	Mixed	1 of 4 probiotic treatment arms w/ significantly less flatulence; 1 w/ sig. less bloating; 2 w/ sig. less diarrhea. No difference in rumbling symptom.
Newcomer (1983)	Not measured	Negative (1 of 1)	
Onwulata (1989)	Negative (1 of 1)	Negative (1 of 1)	
Lin, Savaiano (1991)	Mixed	Negative (7 of 8)	3 of 8 probiotic treatment arms w/ sig. difference in mean breath hydrogen. 1 of 8 probiotic treatment arms eliminated intolerance symptoms "in all subjects."
Lin, Yen (1998)	Mixed	Positive (3 of 4)	2 of 4 probiotic treatment arms w/ sig. difference in mean breath hydrogen.
Savaiano (1984)	Negative (1 of 1)	Negative (1 of 1)	
McDonough (1987)	Mixed	Not measured	1 of 2 probiotic treatment arms w/ sig. difference in mean breath hydrogen.
Kim (1983)	Positive (2 of 3)	Not measured	

Summary (10 studies): Breath hydrogen = 3 positive studies, 3 negative, 3 mixed, 1 not measured. *Expressed as number of probiotic treatment arm results per number of probiotic treatment arms in study. Examples: negative (2 of 3) = 2 negative treatment arms of 3 probiotic treatment arms; Positive 1 of 1 = 1 positive treatment arm of 1 treatment arm.

Table 1.1 Summary of previous clinical trials on LI alleviation effects of probiotics

Cited from (Levri et al., 2005).

1.4 Objective of the present research

β-galactosidase also known as lactase is an enzyme which catalyzes the hydrolytic process of lactose. Because it can be used to lower the amount of lactose in milk for lactose intolerant people or prevent lactose crystallization particularly at low temperature, β-galactosidase is a crucial enzyme in the dairy industry. Furthermore, it has been reported that lactose intolerance (LI) symptoms were alleviated when β-galactosidase was administered to LI subjects, so exogenous β-galactosidases have traditionally attracted much attention as a strategy to manage LI subjects and are currently on the market. For these industrial purposes, β-galactosidases are mainly extracted from microorganisms. The production of β-galactosidases from microbes is a preferred choice because of a higher yield and thus the relatively inexpensive cost of the enzyme. By extension, for β-galactosidase to be used safely in the food industry, microorganisms producing β-galactosidase should be food-grade. From this point of view, bifidobacteria are regarded as a good source of β-galactosidase because of their GRAS (Generally recognized as safe) status.

The aim of the present research was to broaden the application range of β-galactosidase from *Bifidobacterium* by developing

recombinant bifidobacteria that highly express a *Bifidobacterium*-derived β-galactosidase gene and using this recombinant strain in various *in vitro* and *in vivo* studies. The recombinant bifidobacteria were used to efficiently remove lactose in milk and to verify whether the *Bifidobacterium* strains highly-expressing β-galactosidase were effective in alleviating lactose intolerance.

Chapter 2.

Improvement of electroporation-mediated transformation efficiency for a *Bifidobacterium* strain to a reproducibly high level

2.1 Introduction

The human intestine is densely populated by as many as 10^{13} to 10^{14} microorganisms (Suau et al., 1999). Most of these are obligate anaerobes including the Clostridia and Bacteroidia groups and the genus *Bifidobacterium. Bifidobacterium* is one of the dominant organisms in the gut microflora of healthy children and adults comprising more than 1% of the total bacterial count (Blaut et al., 2002; Matsuki et al., 2004a; Matsuki et al., 2004b).

Bifidobacteria are gram−positive, anaerobic, catalase−negative and fermentative bacteria which have beneficial effects on human health and diseases including physiological functions, amelioration of inflammation, immunological responses and resistance to infection (Aoki et al., 2016; Argnani et al., 1996; Jia et al., 2008; Kim and Ji, 2006; Ku et al., 2016; Lee et al., 2002; Mitsuoka, 1990; Sivan et al., 2015; Ventura et al., 2009; You et al., 2004). Because of these advantages, bifidobacteria are considered to be representative probiotics which are defined as live microorganisms that confer a health benefit on the host (Sanders, 2008). Therefore, bifidobacteria are suitable as genetically engineered bacteria to augment human health. However, molecular biological studies on bifidobacteria have been limited by the insufficient genetic tools including effective transformation methods. Even though electroporation, a highly

efficient transformation method, is used for bifidobacteria, the electroporation efficiency of bifidobacteria is usually less than 10^4 CFU / μg DNA in previous studies (Argnani et al., 1996; Guglielmetti et al., 2007; Park et al., 1999b; Rossi et al., 1996; Sangrador-Vegas et al., 2007; Shkoporov et al., 2008).

This study aimed to improve the electroporation-mediated transformation efficiency of bifidobacteria to a reproducibly high level. The crucial factors that determine electroporation efficiency are the restriction-modification system (R-M system), cell wall and cell membrane structure of bacteria. The bifidobacterial electroporation conditions were optimized by focusing on these factors. In addition, I evaluated whether the optimized electroporation methods can be applied to other *Bifidobacterium* species. To the best of my knowledge, this is the first study to use cell wall-weakening agents and a cell membrane permeabilizing agent on bifidobacteria to enhance the electroporation efficiency.

2.2 Materials and methods

2.2.1 Bacterial strains and plasmid DNA

The various species of *Bifidobacterium* and plasmid used in this study are listed in Table 2.1. Bifidobacteria were grown at 37℃ in MRS medium (BD Difco™, Sparks, MD, USA) containing 0.05% L-cysteine·HCl.

The shuttle vector pBES2 was used in this study. pBES2 was constructed from a cryptic plasmid from *Bifidobacterium longum* MG1 ligated to pUC19 and possesses a chloramphenicol-resistance marker (Park et al., 2003).

Table 2.1 Bacterial strains and plasmids used in this study

Bacterial strain	Source or reference
Escherichia coli DH5 α	Lab stock
Bifidobacterium bifidum BGN4	Isolated from breast-fed infant feces (Park et al., 1999c), Lab stock
Bifidobacterium bifidum KCTC 3440	Purchased from Korean Collection for Type Culture
Bifidobacterium bifidum KCTC 3418	Purchased from Korean Collection for Type Culture
Bifidobacterium breve KCTC 3419	Purchased from Korean Collection for Type Culture
Bifidobacterium pseudocatenulatum SJ32	Isolated from healthy human feces (Park et al., 1999c), Lab stock
Bifidobacterium longum RD65	Isolated from healthy human feces, Lab stock
Bifidobacterium longum RD72	Isolated from healthy human feces, Lab stock
Bifidobacterium lactis RD68	Isolated from healthy human feces, Lab stock
Plasmid	**Characteristics**
pBES2	7.6 kbp, Ap^R, Cm^R

2.2.2 DNA manipulation

Plasmid DNA was extracted from *Escherichia coli* DH5α transformants harboring pBES2 with a Plasmid Purification Mini Kit (Nucleogen, Gyeonggi-do, South Korea) and methylated *in vitro* by CpG (M.SssI) and GpC (M.CviPI) methyltransferases (NEB, Ipswich, MA, USA). The amount of DNA was measured by a Qubit 4 Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). To identify the plasmid from the transformants, plasmid DNA was extracted with a Plasmid Purification Mini Kit (Nucleogen) following an initial lysis step. Cells were resuspended in lysis buffer supplemented with lysozyme (20 mg / ml) and incubated at 37℃ for 1 h. The extracted DNA was digested with restriction enzyme *XbaI* (Fermentas, Waltham, MA, USA) and was identified by comparing the restriction patterns with the original *E. coli* derived plasmid DNA.

2.2.3 Preparation of electrocompetent cells

Overnight cultures in MRS broth containing 0.05% L-cysteine·HCl were diluted into 50 ml of fresh MRS broth supplemented with 0.05% L-cysteine·HCl, 0.2 M sucrose (final concentration) and increasing concentrations of NaCl (0.05 – 0.2 M, final concentration) and glycine (0.75 – 1.5%, w/v) for some experiments. The inoculated 50 ml MRS broth with the additives were anaerobically incubated at 37℃ until the OD_{600} reached from 0.2 to 0.7. The bacteria were harvested

by centrifugation and washed three times with 40 ml of electroporation buffer (0.5 M sucrose and 1 mM ammonium citrate, pH 6.0). The final cell pellet was resuspended in 0.5 ml of ice-cold electroporation buffer.

2.2.4 Electroporation

A 0.1-ml of cell suspension was mixed with plasmid DNA and kept on ice for 30 min and then transferred to a pre-cooled Gene Pulser cuvette (Bio-Rad, Hercules, CA, USA). If necessary, cell suspensions mixed with DNA were incubated in the presence of ethanol (0.5 – 2%, v/v) for 10 min shortly before the pulse delivery. The cuvette was pulsed at various field strengths and parallel resistances using the Gene Pulser Xcell Microbial Electroporation System (Bio-Rad). Following the electroporation, 0.9 ml of MRS broth supplemented with 0.05% L-cysteine·HCl and 0.2 M sucrose (final concentration) was added to the bacteria and incubated at 37℃ for 3 h under anaerobic condition. The bacteria were then plated onto MRS agar containing 3 ㎍ / ml of chloramphenicol. The plates were incubated for 36 h under anaerobic condition.

2.3 Results

To enhance the bifidobacterial electrotransformation efficiency, I first chose *Bifidobacterium bifidum* BGN4 as the experimental strain which was isolated from healthy infant. *B. bifidum* BGN4 has been used as a probiotic strain in global food markets since 2001 because of its reported benefits. BGN4 exhibits an outstanding colon cell adhesive ability among other bifidobacteria (Kim et al., 2003; Ku et al., 2009), significant immunoregulatory capacities (Hong et al., 2009; Kim and Ji, 2006; Kim et al., 2007; Lee et al., 2002; Lee et al., 2006) and anti-cancer effects (Ku et al., 2009; You et al., 2004). These features of BGN4 made it as an interesting candidate for the development genetically modified version of bifidobacteria.

In this study, I proceeded with the experiments by applying the optimal conditions of the previous step to the next step. The following sequence of conditions were investigated: 1) *in vitro* methylation, 2) amount of plasmid DNA, 3) electrical parameters, 4) bacterial growth phase, 5) cell wall weakening agent, and 6) cell membrane permeabilizing agent. Each step was repeated three times independently. The transformation efficiency was expressed as the number of transformants per μg of DNA added. Several colonies per plate were randomly selected and the plasmid DNA in the transformants was identified by comparing the restriction patterns

with the original *E. coli* derived plasmid DNA or by sequencing. Results were compared using a nonparametric one-way ANOVA test and post-hoc test (Duncan's multiple range test). $P<0.05$ was considered as statistically significant. Because this study aimed to develop a high-efficiency electroporation protocol, I adopted the experimental method if it showed a consistently high electroporation efficiency in all three experiments, even if it was not statistically significant.

2.3.1 *In vitro* methylation

The restriction-modification (R-M) systems of BGN4 have not been studied in detail. It was investigated which commercial methyltransferase is the most appropriate to protect against the possible R-M system present in BGN4 (Fig. 2.1). GpC methylated plasmid DNA was 8 times more effective than the CpG methylated one, whereas the untreated one yielded no transformants. Double methylated DNA showed no additive effect. This means that BGN4 has an R-M system which can be effectively avoided by *in vitro* GpC methylation rather than by CpG methylation. Therefore, it was decided to use GpC methylated DNA in the next steps of this study. The transformation efficiency of pBES2 isolated from BGN4 and GpC methylated pBES2 was compared to determine how much GpC methylation blocks the R-M systems in BGN4 (Fig. 2.1). pBES2

isolated from BGN4 gave a 5.3 fold higher electroporation frequency as compared to the corresponding GpC methylated plasmid. As a result, GpC methylation does not completely cover the R-M system of BGN4, which reflects the fact that *in vitro* methylation is convenient and effective but may have low versatility (Suzuki, 2012).

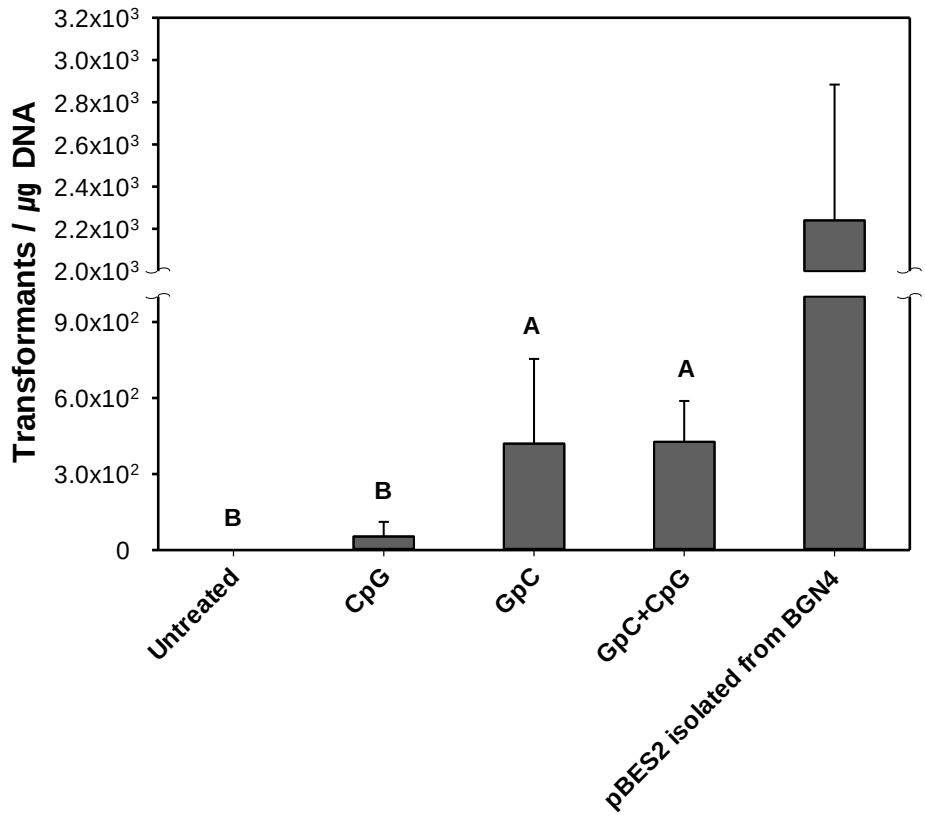


Fig. 2.1 Effect of *in vitro* methylation on the electrotransformation of *B. bifidum* BGN4

Cells were incubated in MRS broth supplemented with 0.05% L-cysteine·HCl and 0.2 M sucrose (final concentration) at 37℃ until an OD_{600} reached 0.4. CpG / GpC / double methylated / untreated plasmid DNA (pBES2) or the corresponding plasmid isolated from BGN4 of 50 ng was added to the competent cell suspension and electroporation was conducted under 12.5 kV / cm field strength, 200 Ω resistance and 25 μF capacitance. Experiments were repeated three times independently. All the groups except pBES2 isolatd from

BGN4 were analyzed by one-way ANOVA. Result for GpC methylated DNA was significantly higher than the results of CpG methylated and untreated DNA.

2.3.2 Amount of plasmid DNA

Various amounts of plasmid DNA were added to the electrocompetent cells to determine the optimal amount of DNA for the electroporation. As the amount of DNA increased, the number of transformants also tended to increase, but the transformation efficiency was not the same (Fig. 2.2.B). The largest number of transformants was obtained from the largest amount of DNA (1 μg) used in this study and the greatest electrotransformation efficiency was obtained when 50 ng of plasmid DNA was used. To minimize the amount of time and work and maximize the electrotransformation efficiency, it was decided to use 50 ng of DNA. Although the standard deviation is high, this is the early stage of the optimization; thus, many factors that affect the transformation efficiency were not yet controlled for, and there are batch-to-batch variations in the electroporation process. In addition, because the transformation efficiency should be adjusted to 1 ug DNA, as the amount of inserted DNA becomes smaller, the standard deviation becomes greater. The raw data show that the tendencies between each independent experiment are not different (Fig. 2.2.A).

A

Amount of plasmid DNA	Expt. 1		Expt. 2		Expt. 3	
	Transformants	Transformation efficiency (CFU / μg DNA)	Transformants	Transformation efficiency (CFU / μg DNA)	Transformants	Transformation efficiency (CFU / μg DNA)
1 ng	1	1,000	0	0	0	0
50 ng	48	960	10	200	39	780
100 ng	55	550	12	120	53	530
500 ng	227	454	54	108	157	314
1,000 ng	500	500	175	175	160	160

B

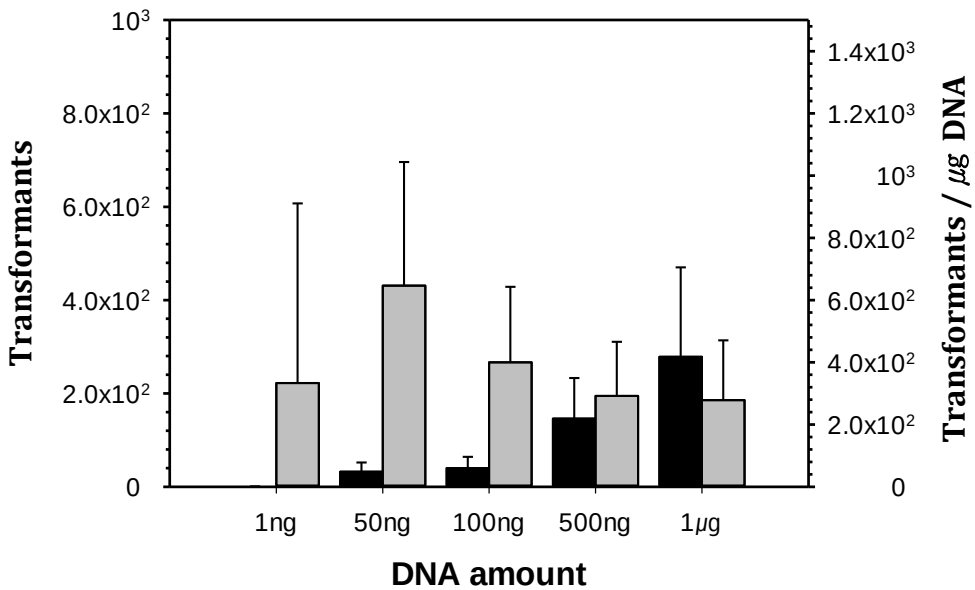


Fig. 2.2 Effect of amount of plasmid DNA on the electrotransformation of *B. bifidum* BGN4

Cells were incubated in MRS broth supplemented with 0.05% L-cysteine·HCl and 0.2 M sucrose (final concentration) at 37℃ until an OD_{600} reached 0.4. Separately, 1, 50, 100, 500, 1,000 ng of GpC methylated plasmid DNA (pBES2) was added to the competent cell suspension and electroporation was conducted under 12.5 kV / cm field strength, 200 Ω resistance and 25 μF capacitance. Total number of transformants (dark bar) and electrotransformation efficiency (light bar) were quantified. Experiments were repeated three times independently.

2.3.3 Electrical parameters

The optimization of the electroporation process involves several important factors. The three main parameters are the wave form, which is the pulse shape, the field strength and the pulse length. The field strength is measured as the voltage delivered across an electrode gap and presented as the applied voltage divided by the gap size of the electroporation cuvette. The pulse length is the duration of time that the sample is exposed to the pulse. These parameters should be optimized for different species and even strains (Löfblom et al., 2007). The optimal electrical condition of *B. bifidum* BGN4 was found by adjusting these parameters. The Gene Pulser Xcell Microbial Electroporation System (Bio-Rad, USA) used in my laboratory supports three wave forms: a square wave pulse, exponential decay wave pulse, and time-constant pulse. Among these, the exponential decay wave pulse is routinely used for electroporation of bacteria. When it is used, the voltage rises rapidly to the peak voltage set then decreases over time. The pulse length in an exponential decay wave pulse is called the time constant which is not the same as the time-constant pulse and is defined as the time required for the voltage of the untruncated pulse to decline to 1 e^{-1} (~37%) of the peak amplitude (Löfblom et al., 2007; Shigekawa and Dower, 1988). The time constant is modified by adjusting the resistance and capacitance values. Hence, the optimum electrical

conditions was found by adjusting the field strength and resistance while using the exponential decay wave pulse. The effects on the transformation efficiency for field strengths of 12.5 and 15 kV / cm and resistances between 50 and 600 Ω were evaluated. The best efficiency was obtained under a field strength of 15 kV / cm and a resistance of 200 Ω, which is approximately 2 times higher than that of the existing conditions used in the laboratory with a field strength of 12.5 kV / cm and a resistance of 200 Ω (Table 2.2).

Table 2.2 Effect of electrical parameters on the electrotransformation of *B. bifidum* BGN4.

Cells were incubated in MRS broth supplemented with 0.05% L-cysteine·HCl and 0.2 M sucrose (final concentration) at 37℃ until an OD_{600} reached 0.4. GpC methylated plasmid DNA (pBES2) of 50 ng was added to the competent cell suspension and electroporation was conducted under indicated field strength and resistance at 25 ㎌ capacitance. Experiments were repeated three times independently.

Field strength (kV / cm)		Resistance (Ω)							
		50	100	150	200	300	400	500	600
12.5	Time constant (mS)	1.3	2.6	3.6	4.7	5.2	6.2	7.7	7.9
	Transformation efficiency (transformants / ㎍ DNA)	387	533	160	2,680	2,330	113	2,040	987
15	Time constant (mS)	1.3	2.5	3.7	4.7	6.5	7.9	8.5	9.8
	Transformation efficiency (transformants / ㎍ DNA)	1,040	2,350	3,540	4,810	2,290	607	53	40

2.3.4 Bacterial growth phase

The effects of the bacterial growth phase on the transformation efficiency was investigated. Overnight-grown BGN4 were inoculated into 50 ml of MRS broth supplemented with 0.05% L-cysteine·HCl and 0.2 M sucrose (final concentration) and cultured until the OD_{600} reached from 0.2 to 0.7. when the OD_{600} was 0.4 (early-exponential phase), the transformation frequency was notably increased, which is 2.9 times higher than the second highest efficiency of OD_{600} 0.3 and 15 times higher than the lowest efficiency of OD_{600} 0.7 (Fig. 2.3).

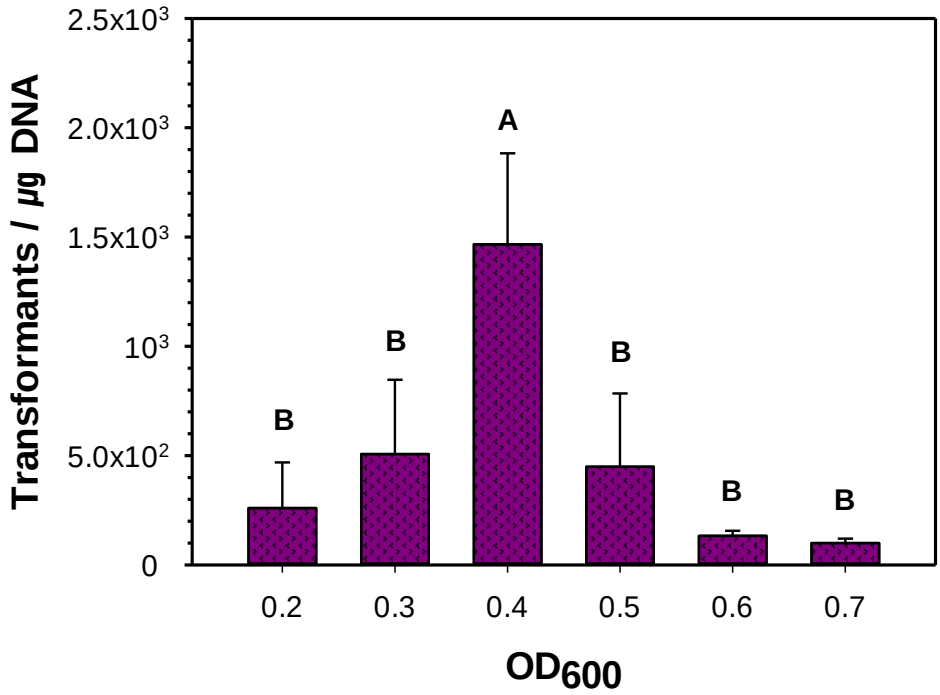


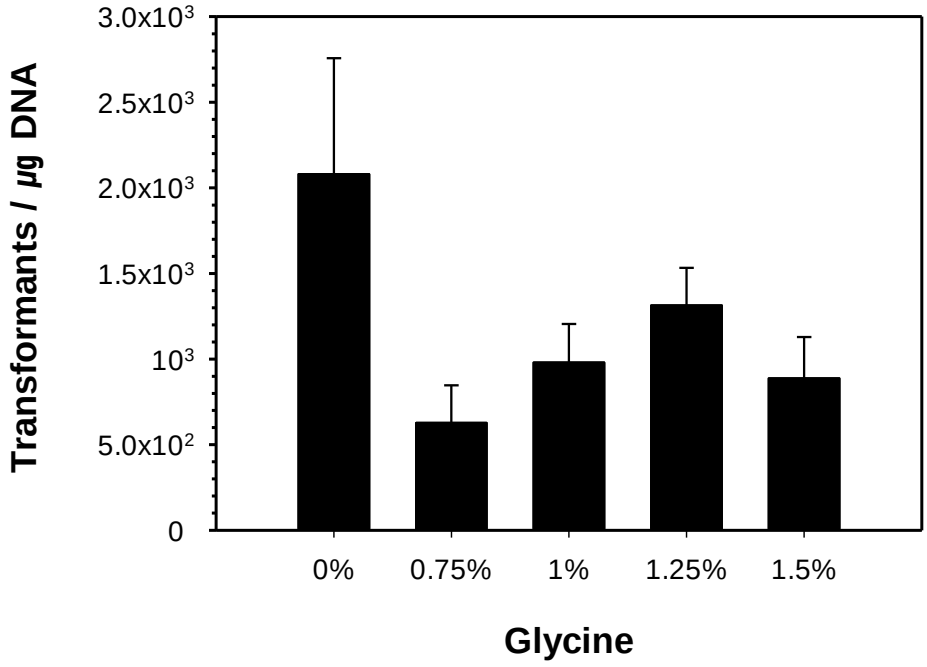
Fig. 2.3 Effect of bacterial growth phase on the electrotransformation of *B. bifidum* BGN4

Cells were incubated in MRS broth supplemented with 0.05% L-cysteine·HCl and 0.2 M sucrose (final concentration) at 37℃ until an OD_{600} reached from 0.2 to 0.7. GpC methylated plasmid DNA (pBES2) of 50 ng was added to the competent cell suspension and electroporation was conducted under 15 ㎸ / cm field strength, 200 Ω resistance and 25 ㎌ capacitance. Experiments were repeated three times independently. Result for OD_{600} 0.4 was statistically significantly higher (by one-way ANOVA) than the results of the others.

2.3.5 Cell wall weakening agent

Cell wall weakening agents were added to the medium to investigate their effects on the electroporation efficiency. Glycine has been frequently shown to increase the electroporation efficiency in some bacteria (Gerber and Solioz, 2007; Hashiba et al., 1990; Holo and Nes, 1989; Kim et al., 2005; Pyne et al., 2013; Thompson and Collins, 1996), and NaCl was used in one study (Palomino et al., 2010). Based on this, glycine and NaCl were each tested to determine whether these additives enhance the electroporation efficiency of *B. bifidum* BGN4. The highest glycine and NaCl concentrations were selected as 1.5% and 0.2 M, respectively, to minimize the inhibition of the bacterial growth and the experiments were performed at lower concentrations accordingly. When 0.2 M NaCl was used to treat the cells, an approximately 20−fold increase in the electroporation frequency was obtained compared to the control without any treatment (Fig. 2.4.B). However, glycine, already known to be effective for several bacteria, did not enhance transformation in this study with *B. bifidum* BGN4 (Fig. 2.4.A).

A


3.0x10³
2.5x10³
2.0x10³
1.5x10³
10³
5.0x10²
0
Transformants / µg DNA
0%
0.75%
1%
1.25%
1.5%
Glycine

B

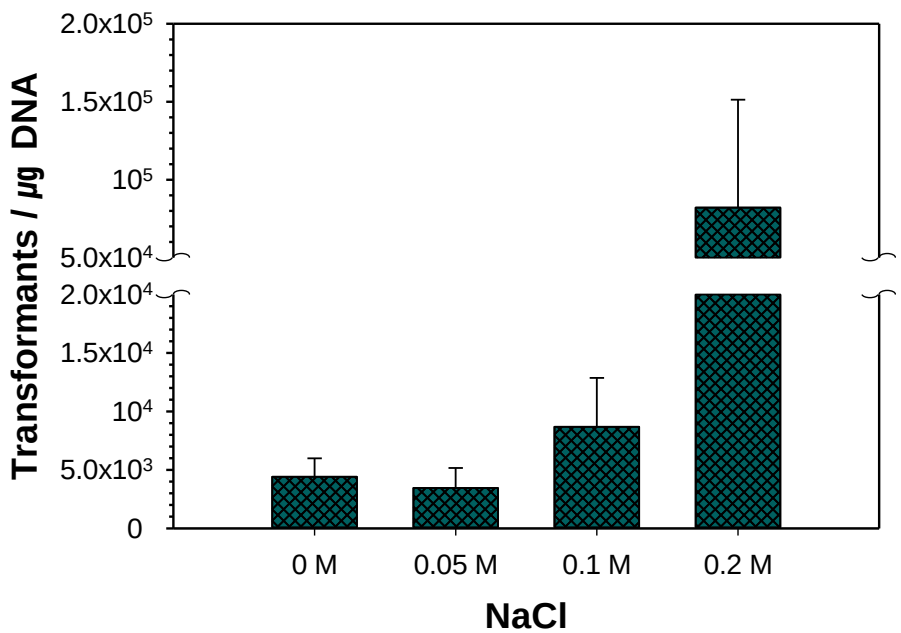

2.0x10⁵
1.5x10⁵
10⁵
5.0x10⁴
2.0x10⁴
1.5x10⁴
10⁴
5.0x10³
0
Transformants / µg DNA
0 M
0.05 M
0.1 M
0.2 M
NaCl

Fig. 2.4 Effect of cell wall weakening agents on the electrotransformation of *B. bifidum* BGN4

Cells were incubated in MRS broth supplemented with 0.05% L-cysteine·HCl, 0.2 M sucrose (final concentration) increasing concentrations of NaCl (0 – 0.2 M, final concentration) or glycine (0 – 1.5%) at 37℃ until an OD_{600} reached 0.4. GpC methylated plasmid DNA (pBES2) of 50 ng was added to the competent cell suspension and electroporation was conducted under 15 ㎸ / cm field strength, 200 Ω resistance and 25 ㎌ capacitance. A. Investigation of glycine concentrations. B. Investigation of NaCl concentrations. Experiments were repeated three times independently.

2.3.6 Cell membrane permeabilizing agent

Finally, it was investigated whether ethanol which is known to be effective with some species of bacteria (Assad-García et al., 2008; Pyne et al., 2013; Sharma et al., 2007) improves the electrotranformation efficiency by altering the properties of the cell membrane. Because ethanol tolerance varies depending on the strain (Gold et al., 1992), the tolerance of *B. bifidum* BGN4 was examined by inoculating this strain with MRS medium containing various concentrations of ethanol. Because the growth of BGN4 was considerably retarded at above 2% (v/v) ethanol, the effect of ethanol on the electroporation efficiency was investigated at lower concentrations. Ten minutes prior to the electroporation, ethanol was added to a cell-DNA suspension. In the ethanol-treated groups, the electroporation efficiency generally tended to be higher than that in the untreated group. Among them, the 2% ethanol-treatment provided a 1.7-fold increase in the electroporation efficiency compared with the no ethanol treatment (Fig. 2.5).

During this study, the conditions for electroporation-mediated transformation of *Bifidobacterium* were optimized. The optimized conditions drastically improved the electrotransformation efficiency from 10^3 to 10^5 which is about a 72-fold increase from the existing conditions in my laboratory (Table 2.3). The differences between the initial method and the optimized method are the use of cell wall

weakening agent and cell membrane permeabilizing molecule and the electrical parameter change, thereby these factors are key points to increase the electroporation efficiency of BGN4. Among them, using the optimization scheme employed herein, the cell wall weakening mediated by NaCl is the most significant factor, which improves the electroporation frequency by 20 times. In order to identify whether these methods can be applied to other *Bifidobacterium* species, two *B. bifidum* strains, *B. breve*, *B. pseudocatenulatum*, two *B. longum* strains and *B. lactis* were assayed. As a result, the electroporation efficiencies increased from 2.5 to 14 times as compared with the initial method and the transformants appeared in all tested species even the species which were not transformed under the initial method (Table 2.4).

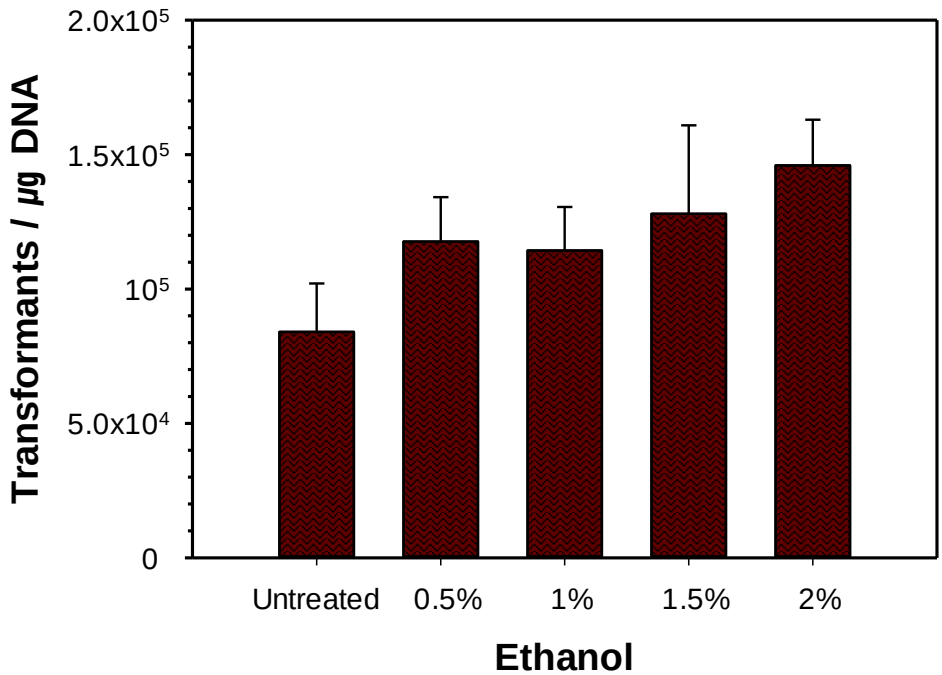


Fig. 2.5 Effect of cell membrane permeabilizing agent on the electrotransformation of *B. bifidum* BGN4

Cells were incubated in MRS broth supplemented with 0.05% L-cysteine·HCl, 0.2 M sucrose (final concentration) and 0.2 M NaCl (final concentration) at 37℃ until an OD_{600} reached 0.4. GpC methylated plasmid DNA (pBES2) of 50 ng was added to the competent cell suspension and the DNA-cell suspensions were supplemented with 0.5, 1, 1.5 or 2% ethanol (v/v) ten minutes prior to pulse delivery. Electroporation was conducted under 15 ㎸ / cm field strength, 200 Ω resistance and 25 ㎌ capacitance. Experiments were repeated three times independently.

Table 2.3 Comparison between optimized high-level electroporation method and initial method for *B. bifidum* BGN4

Condition	Initial method	Optimized method
Bacterial growth phase	OD_{600} 0.4	OD_{600} 0.4
Cell wall weakening agent added to medium	–	0.2 M NaCl
Amount of plasmid DNA	50 ng	50 ng
Types of DNA methylation	GpC methylation	GpC methylation
Cell membrane permeabilizing agent added to cell-DNA suspension	–	2% Ethanol
Electrical parameters	12.5 kV / cm, 25 μF, 200 Ω	15 kV / cm, 25 μF, 200 Ω
Transformation efficiency (CFU / μg DNA)	2.04×10^3	1.46×10^5

Table 2.4 Comparison of electroporation efficiency of other *Bifidobacterium* species between initial and optimized method

Bacterial strain	Initial method	Optimized method
	Transformation efficiency (CFU / μg DNA)	Transformation efficiency (CFU / μg DNA)
B. bifidum KCTC 3440	$1.3 \pm 1.2 \times 10^1$	$3.3 \pm 2.3 \times 10^1$
B. bifidum KCTC 3418	0	$1.4 \pm 0.7 \times 10^2$
B. breve KCTC 3419	0	$7.3 \pm 2.3 \times 10^1$
B. pseudocatenulatum SJ32	$0.7 \pm 1.2 \times 10^1$	$1.0 \pm 0.4 \times 10^2$
B. longum RD65	0	$1.3 \pm 1.2 \times 10^1$
B. longum RD72	$2.0 \pm 1.7 \times 10^1$	$8.7 \pm 5.0 \times 10^1$
B. lactis RD68	$8.9 \pm 8.5 \times 10^3$	$4.3 \pm 0.3 \times 10^4$

2.4 Discussion

Electroporation-mediated transformation is generally more efficient than chemotransformation and is widely used for gram-positive and gram-negative bacteria (Itoh et al., 1994; Iwasaki et al., 1994; Wirth et al., 1989). The average electroporation efficiency is 10^4 – 10^5 CFU / ㎍ DNA and even up to 10^{10} CFU / ㎍ DNA for *E. coli* (Aune and Aachmann, 2010; Dower et al., 1988; Hanahan et al., 1991). However, bifidobacteria are vulnerable to oxygen and have a multilayered and complex cell wall (FISCHER et al., 1987) as well as restriction-modification systems making it difficult to acquire a high electroporation efficiency (Serafini et al., 2012). Indeed, the electroporation efficiency of bifidobacteria was usually less than 10^4 CFU / ㎍ DNA in previous studies (Argnani et al., 1996; Guglielmetti et al., 2007; Park et al., 1999b; Rossi et al., 1996; Sangrador-Vegas et al., 2007; Shkoporov et al., 2008). Such a lack of efficient transformation methods has limited molecular-level studies and food-grade vector development of bifidobacteria. Therefore, I focused on finding highly efficient electroporation protocols for bifidobacteria in this study. First, a suitable strain of bifidobacteria which accepts plasmid DNA was chosen. *B. bifidum* is predominant in the gut population of healthy breast-fed infants (Milani et al., 2013; Turroni et al., 2012). Because it colonizes initially the infantile

intestine, *B. bifidum* exists widely among the intestinal bifidobacterial population in healthy adults (Ku et al., 2016; Turroni et al., 2014). Among these bacterial strains, *B. bifidum* BGN4 originates from the feces of a breast-fed infant and is widely used as a probiotic strain in global food markets because of its reported benefits (Hong et al., 2009; Kim et al., 2003; Kim and Ji, 2006; Kim et al., 2007; Ku et al., 2009; Lee et al., 2002; Lee et al., 2006; You et al., 2004). In this respect, it was decided to use *B. bifidum* BGN4 as the experimental strain.

The important factors determining the electroporation efficiency are the R-M system, cell wall and cell membrane of a bacteria, so I focused on them. At first, there was an attempt to overcome the R-M systems of BGN4. R-M systems of several *Bifidobacterium sp.* have been determined by classical restriction analysis (Hartke et al., 1996; Khosaka et al., 1983; Khosaka et al., 1982; Kim et al., 2010; O'Connell Motherway et al., 2009) and by methylome analysis generated by single molecule real-time (SMRT) sequencing (Bottacini et al., 2017; Mary et al., 2014; O' Callaghan et al., 2015). However, the R-M systems of BGN4, even *B. bifidum*, have not been studied in detail to my knowledge. There are various strategies to block the R-M system of a transformation host: *in vitro* methylation using methyltransferases, *in vivo* methylation by expressing methyltransferase genes in *E. coli*, and removal of specific plasmid sites recognized by RM systems (Suzuki, 2012). *In vitro* methylation

using methyltransferases, the most convenient method, was attempted and it was found that the R-M system of BGN4 was effectively blocked by GpC methylation. GpC methylated plasmid DNA was 8 times more effective than the CpG methylated one, whereas the untreated one yielded no transformants. Double methylated DNA showed no additive effect (Fig. 2.1). This means that the R-M system of BGN4 is largely affected by *in vitro* GpC methylation rather than by CpG methylation. The transformation frequency of pBES2 isolated from BGN4 and GpC methylated pBES2 was compared to determine how much GpC methylation blocks the R-M systems in BGN4. There was a 5.3 fold higher electroporation efficiency for pBES2 isolated from BGN4 compared to the corresponding GpC methylated plasmid (Fig. 2.1). As a result, because GpC methylation does not completely cover the R-M systems of BGN4, applying additional *in vitro* adenine methylation or *in vivo* methylation by expressing methyltransferase genes in *E. coli* is expected to yield a higher efficiency. The putative R-M systems in BGN4 were additionally analyzed through the REBASE website (http://rebase.neb.com/rebase) using the genome sequence of BGN4. Notably, the majority of the predicted recognition sites include the sequence where CpG methylation could work rather than GpC methylation. This is in contrast to the result shown in Fig. 2.1. Among the putative R-M systems of BGN4, there are several systems in which the recognition sequence is not predicted. I cautiously suppose

that these less predicted R-M systems have a recognition site where GpC methylation can act and are relatively active in BGN4. To clarify this, it will be necessary to perform a methylome analysis of BGN4 through single molecule real-time (SMRT) sequencing.

The initial electrical parameters which were the field strength and the resistance were next adjusted. The electroporation efficiency nearly doubled when the field strength was increased from 12.5 to 15 kV / cm at 200 Ω (Table 2.2), which is consistent with the studies that higher electric field strengths resulted in greater transformation efficiencies for *Campylobacter jejuni* (Miller et al., 1988) and for bifidobacteria (Argnani et al., 1996; Serafini et al., 2012) as long as the lethality does not exceed the threshold level. High-voltage pulses during electroporation are likely to destabilize the cell walls of gram-positive bacteria (Pyne et al., 2013; Trevors et al., 1991). Indeed, the electroporation efficiency tended to increase overall at 15 kV / cm than at 12.5 kV / cm at resistances ranging from 50 to 400 Ω. However, once the resistance was 500 Ω or above which means the time constant is increased by that much, the efficiency was the opposite because of the excessive lethality (Table 2.2).

The electroporation efficiency is largely dependent on the structure and density of the cell wall (Aune and Aachmann, 2010); thus, increasing the fragility of the cell wall by using cell wall weakening agents usually improves the transformation efficiency (Gerber and

Solioz, 2007; Hashiba et al., 1990; Holo and Nes, 1989; Kim et al., 2005; Palomino et al., 2010; Pyne et al., 2013; Thompson and Collins, 1996). In this study, glycine and NaCl were used as the cell wall weakening agents. The mechanisms by which these additives weaken the cell wall are somewhat different. Glycine replaces D- and L-alanine residues in peptidoglycan layers, weakening the cell wall due to reduction of cross-linking (Hammes et al., 1973). NaCl reduces peptidoglycan interpeptide bridges probably by interfering with the glycine addition process during peptidoglycan synthesis and results in loose cross-linking of the cell wall (Palomino et al., 2009; Vijaranakul et al., 1995). A limitation of using these agents is that they can be toxic to the cells depending on the species and strain (Aune and Aachmann, 2010). Therefore it is critical to find the optimal concentration of these additives to increase the electroporation efficiency without inhibiting the cell growth. Glycine and NaCl were added to the extent that they did not completely inhibit the cell growth, and NaCl, rather than glycine, worked well in increasing transformation frequency in BGN4 (Fig. 2.4). Although glycine is a widely used substance for increasing the electroporation efficiency of gram-positive bacteria, it did not increase the frequency in BGN4 at all. On the other hand, the use of NaCl as a cell wall weakening agent in electroporation has been reported rarely but it was effective for bifidobacteria. Because the structure of the cell wall is different for each species and strain, empirical screening for

the type and concentration of a cell wall weakening agent suitable for the bacteria used in the experiments is necessary.

The cell membrane also acts as a physical obstacle during electroporation (Pyne et al., 2013). It has been reported that the addition of ethanol to cells increases the efficiency of electroporation (Assad-García et al., 2008; Pyne et al., 2013; Sharma et al., 2007). It is well known how ethanol acts on the cell membrane. Ethanol binds to the lipid–water interface of the phospholipid bilayers and weakens the hydrophobic barrier of the membrane, thereby increasing the fluidity and pore size of it (Barry and Gawrisch, 1994; Baskaran et al., 1995; Ingram, 1986; Weber and de Bont, 1996). Because ethanol is also toxic to cells at certain concentrations and ethanol tolerance varies depending on the strain, the minimum inhibitory concentration (MIC) of ethanol against BGN4 was first examined. Because BGN4 growth was inhibited at an ethanol concentration above 2% (v/v), the effect of ethanol on the electroporation efficiency was investigated at lower concentrations. Though it is not as dramatic as the cell wall weakening agent, the permeabilization of the cell membrane with ethanol slightly increased the electroporation efficiency (Fig. 2.5).

As a result, the electroporation–mediated transformation efficiency of *B. bifidum* BGN4 increased from 10^3 to 10^5 CFU / ㎍ DNA (Table 2.3). The optimized electrotransformation conditions were extensively applied to other *Bifidobacterium* species; two *B. bifidum* strains, *B. breve*, *B. pseudocatenulatum*, two *B. longum*

strains and *B. lactis.* The electroporation efficiencies increased from 2.5 to 14 times as compared with the initial method and the transformants appeared in all tested species even the species which were not transformed under the initial method (Table 2.4). The differences between the initial method and the optimized method are the cell wall weakening agent and cell membrane permeabilizing agent treatment and the electrical parameter change, thereby these factors are key points to increase the electroporation efficiency of all tested *Bifidobacterium* species. To the best of my knowledge, this is the first study to apply cell wall-weakening agents and a cell membrane permeabilizing agent to bifidobacteria to enhance the electroporation efficiency. It is expected that further optimizing the concentrations of the additives in these newly optimized electroporation conditions considering the diversity of the cell walls and membrane structures of the *Bifidobacterium* strains would contribute to improvement of other bifidobacterial electroporation efficiencies.

Chapter 3.

Cloning and heterologous expression of the β-galactosidase gene from *Bifidobacterium longum* RD47 in *B. bifidum* BGN4

3.1 Introduction

β-galactosidase (lactase, EC 3.2.1.23) catalyzes the hydrolytic process of the terminal β-1,4-D-galactosyl residues found in β-D-galactosides, such as lactose, and releases D-glucose and D-galactose as an end product (Kamran et al., 2016; Nguyen et al., 2015; Yuan et al., 2008). Because it can be used to lower the amount of lactose in milk for lactose intolerant people or prevent lactose crystallization particularly at low temperature, β-galactosidase is a crucial enzyme in the dairy industry (Gänzle et al., 2008; Nguyen et al., 2006; Nguyen et al., 2015; Sani et al., 1999; Splechtna et al., 2007). In addition to the hydrolysis activity, some of these enzymes can be used to produce galactooligosaccharides (GOS), widely utilized as a prebiotics product, through the transgalactosylation reaction (Nguyen et al., 2012). For these industrial purposes, β-galactosidases are mainly extracted from microorganisms including *Bifidobacterium* (Hsu et al., 2007; Møller et al., 2001) and especially from fungi. The production of β-galactosidase from microbes is a preferred choice because of a higher yield and thus the relatively inexpensive cost of the enzyme. By extension, in order for β-galactosidase to be used safely in the food industry, microorganisms producing β-galactosidase should be food-grade. From this point

of view, bifidobacteria are regarded as a good source of β-galactosidase because of their GRAS (Generally recognized as safe) status (Chanalia et al., 2018).

Bifidobacteria are representative of probiotics defined as non-pathogenic microorganisms that provide a health benefit to the host (Lewis et al., 2016). Since their first discovery by Tissier, these microorganisms have been extensively investigated by some scientists. Bifidobacteria are saccharolytic microorganisms, and the saccharolytic metabolism of bifidobacteria is associated with carbohydrate degrading enzymes, particularly glycosyl hydrolases, which enable the use of a variety of glycan substrates available in the intestines directly or indirectly (Koropatkin et al., 2012; Milani et al., 2015; Rivière et al., 2016; Turroni et al., 2018). Among the glycosyl hydrolases encoded by bifidobacteria, β-galactosidase enables bifidobacteria to utilize (human) milk and milk-based substrates, i.e., lactose, human milk oligosaccharide (HMO) and GOS (James et al., 2016; O'Connell Motherway et al., 2013). The hydrolysis and transgalactosylation properties of β-galactosidase for milk-based substrates have been studied in several bifidobacteria to date (Møller et al., 2001; Roy et al., 1994; Smart et al., 1993; Van Laere et al., 2000).

Recently, the β-galactosidase activities of 43 lactic acid bacteria were assessed. Among them, *Bifidobacterium longum* RD47 was found to be one of the bifidobacterial strains with the greatest level of β-galactosidase activity (Han et al., 2014). The whole genome sequence of RD47 was identified, and there were three β-galactosidase genes (G1, G3 and G4) in it. The properties of hydrolysis and transgalactosylation were examined for each gene, and it was revealed that G1 showed the greatest contribution to the hydrolysis and transgalactosylation activity of RD47. The optimal pH and temperature for the hydrolytic activity of G1 is pH 8.5 and 40℃, respectively, which are moderate for activity in the human colonic environment (Oh et al., 2017). Furthermore, G1 yielded maximum production of β-GOS (Oh et al., 2017) and an oligosaccharide with a new structure (data not shown) at pH 8.5 and 45℃.

The aim of this study was to obtain a new recombinant bifidobacteria with significant β-galactosidase expression by inserting several bifidobacterial promoters upstream of the G1. The recombinant bifidobacteria were used to remove lactose in milk in this study and will be used in a future study to determine whether the alleviation of lactose intolerance depends on the β-galactosidase activity of bifidobacteria. Incidentally, the strength and characteristics of several bifidobacterial promoters which are known to be constitutively strong shown by previous studies were compared.

3.2 Materials and Methods

3.2.1 Bacterial strains, plasmid DNA and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 3.1. *E. coli* were grown at 37℃ in Luria-Bertani (LB) medium (BD Difco™, Sparks, MD, USA) with shaking at 150 rpm. Bifidobacteria were cultured at 37℃ in Man Rogosa Sharpe (MRS) medium (BD Difco™) containing 0.05% L-cysteine·HCl.

Antibiotics were used at the following concentrations: 100 μg/ml ampicillin (BIO BASIC INC., Markham, ON, Canada) for *E. coli* and 3 μg/ml chloramphenicol (Duchefa, Haarlem, The Netherlands) for bifidobacteria.

Table 3.1 Bacterial strains and plasmids used in this study

Strains and Plasmids	Description	Source or reference
Bacterial strains		
Bifidobacterium bifidum BGN4	wild type; Transformation host; Original source of P919	Isolated from breast-fed infant feces (Park et al., 1999b)
B. longum RD47	wild type; Original source of G1	Isolated from breast-fed infant feces
B. pseudocatenulatum SJ32	wild type; Original source of P504	Isolated from healthy human feces (Park et al., 1999c)
B. lactis SH5	wild type; Original source of P572	Isolated from healthy human feces
Escherichia coli DH5 α	φ80dlacZΔM15 Δ(lacZY-argF)U169 deoR recA1 endA1 hsdR17(r_K-m_K+) sup E44 thi-1 gyrA96 relA1; Cloning host	Lab stock

BGN4+pBES2	*B. bifidum* BGN4 harboring pBES2	This work
BGN4+G1	*B. bifidum* BGN4 harboring pB-G1	This work
BGN4+P504G1	*B. bifidum* BGN4 harboring pB-P504G1	This work
BGN4+P572G1	*B. bifidum* BGN4 harboring pB-P572G1	This work
BGN4+P919G1	*B. bifidum* BGN4 harboring pB-P919G1	This work
B. lactis AD011	Lactic acid bacteria in nature	Lab stock
B. lactis RD68		
B. longum RD65		
B. longum RD72		
B. longum BORI		
B. longum RD03		
B. pseudocatenulatum INT57		
Lactobacillus casei KFRI 699		
L. plantarum KFRI 708		
L. sakei KOK		

B. bifidum KCTC 3418	
B. breve KCTC 3419	
B. bifidum KCTC 3440	
B. pseudocatenulatum G4 KCTC 3223	
B. angulatum KCTC 3236	
B. catenulatum KCTC 3221	
B. infantis KCTC 3249	
B. breve KCTC 3220	Purchased from Korean Collection for Type Culture
B. bifidum KCTC 3202	
L. delbrueckii bulgaricus KCTC 3635	
L. rhamnosus KCTC 3237	
L. acidophilus KCTC 3168	
Streptococcus thermophilus KCTC 3779	
Streptococcus thermophilus KCTC 5092	
Lactococcus lactis KCTC 2013	

B. breve ATCC 15700 *B. adolescentis* ATCC 15703 *B. animalis* ATCC 25527 *Leuconostoc mesenteroides* ATCC 27258 *Lactococcus cremoris* ATCC 19257		Purchased from American Type Culture Collection
B. thermophilum KCCM 12097		Purchased from Korean Culture Center of Microorganisms
Plasmids		
pBES2	7.6kbp, Ap^R, Cm^R, *Bifidobacterium – E. coli* shuttle vector	(Park et al., 2003)
pB-G1	11kbp, pBES2 derivate, containing PG1-G1 expression cassette	This work
pB-G1ORF	10.7kbp, pBES2 derivate, containing G1ORF	This work
pB-P504G1	11kbp, pBES2 derivate, containing P504-G1 expression cassette	This work

pB-P572G1	11.2kbp, pBES2 derivate, containing P572-G1 expression cassette	This work
pB-P919G1	10.9kbp, pBES2 derivate, containing P919-G1 expression cassette	This work

3.2.2 DNA molecular cloning and transformation

The target DNA fragments were amplified using TAKARA PrimeSTAR GXL DNA Polymerase (TAKARA, Kusatsu, Japan), and each primer set is listed in Table 3.2. First, the DNA fragment encoding the β-galactosidase, G1, was PCR-amplified from *B. longum* RD47 and cloned into pBES2 via *XbaI* and *EcoRI* to construct pB-G1. Second, the DNA fragment encoding the β-galactosidase open reading frame (ORF) without the promoter, G1ORF, was PCR-amplified from RD47 and cloned into pBES2 via *KpnI* and *EcoRI* to construct pB-G1ORF. To investigate the regulatory effects of the bifidobacterial promoter, regions of different gene promoters, P504, P572 and P919, were amplified from *B. pseudocatenulatum* SJ32, *B. lactis* SH5 and *B. bifidum* BGN4, respectively. The amplified putative promoter sequences were cloned into the *XbaI* and *KpnI* sites of pB-G1ORF to incorporate the promoter upstream from the β-galactosidase.

Successful cloning was confirmed by nucleotide sequencing. Each sub-cloned vector was introduced into *E. coli* via heat-shock and the $CaCl_2$ method and introduced into *B. bifidum* BGN4 by electroporation with the Gene Pulser Xcell Microbial Electroporation System (Bio-Rad, Hercules, CA, USA). The positive colonies were isolated, and the plasmids were purified and identified by restriction and sequence analyses.

Table 3.2 Primers for amplifying the β-galactosidase gene and putative promoters from *Bifidobacterium*

Primer name	Sequences of primer		Product	Product information
	Forward (5'→3')	Reverse (5'→3')		
primer P504	TtctagaGGGCGATTTTCTGCAG GGTT	GggatccACGTTCCTCTTTCGCATC	P504	A promoter of β-glucosidase gene in SJ32 (Youn et al., 2012)
primer P572	TtctagaATGCTGCTCCTTATGT GTC	CggatccTGCTGATTCCTCCTGTCG	P572	A promoter of β-glucosidase gene in SH5 (Youn et al., 2012)
primer P919	TtctagaTGAAGTGTGTCGTGT GGC	CggatccTGGTGTACCTTTTCTTG CTT	P919	A promoter of ribosomal protein gene in BGN4 (Wang et al., 2012)
primer G1ORF	TggatccATGGCCGAGAACACC TCG	CgaattcTCAGCTTAACGCCACAGC	G1ORF	A β-galactosidase gene in RD47

primer G1	CGtctagaCCTTCTCCCTTAAATTTTTCTC	CcaattgTCAGCTTAACGCCACAGC	PG1+G1ORF	A β-galactosidase gene in RD47

3.2.3 DNA manipulation

The chromosomal DNA of each bacterial strain used in this study was isolated with the MG^{TM} Cell Genomic DNA Extraction SV Kit (MGmed, Seoul, South Korea). Plasmid DNA was extracted from *Escherichia coli* DH5α transformant harboring pBES2 or each sub-cloned vector with a Plasmid Purification Mini Kit (Nucleogen, Gyeonggi-do, South Korea) and methylated *in vitro* by GpC (M.CviPI) methyltransferase (NEB, Ipswich, MA, USA). To identify the plasmid from the *B. bifidum* BGN4 transformants, plasmid DNA was extracted with a Plasmid Purification Mini Kit (Nucleogen) following an initial lysis step. Cells were resuspended in lysis buffer supplemented with lysozyme (20 mg / ml) and incubated at 37℃ for 1 h. The extracted DNA was identified by sequencing and comparing the restriction patterns with the original plasmid DNA derived from *E. coli*.

3.2.4 Enzyme preparation and assay

The incubated bacteria were centrifuged (10,000 ×g for 3 min at 4℃), and the harvested pellet was washed with 50 mM sodium phosphate buffer (pH 7.0). The washed cells were resuspended in the phosphate buffer (pH 7.0) and disrupted with a sonicator (Q500; K-corporation, Gyeonggi-do, South Korea) for 1.0 s with 1.0 s off intervals for 10 min in *E. coli* and 15 min in bifidobacteria. The

disrupted bacterial solution was centrifuged at 10,000 ×g for 10 min at 4℃, and the supernatant was used as a crude enzyme extract to assay the β-galactosidase activity.

The relative enzyme activity was measured by the release of para-nitrophenol from para-nitrophenyl (ρNP) β-D-galactopyranoside (Sigma Aldrich, St. Louis, MO, USA). 80 μℓ of the crude enzyme extract were added to 20 μℓ of 5 mM ρNP β-D-galactopyranoside suspended in a 50 mM sodium phosphate buffer (pH 7.0). The mixture was incubated at 37℃, and the reaction was stopped by adding 100 μℓ of 1 M Na_2CO_3. The activity was measured in a 96-well microplate at 405 nm with a spectrophotometer (Model 680 Microplate reader; Bio-Rad, Hercules, CA, USA). One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of ρNP per minute at 37℃ and pH 7.0. The volumetric activity (enzyme activity level relative to ml fermentation broth) was determined as units of β-galactosidase activity. The protein concentration was calculated according to the Bradford method (Bradford, 1976).

3.2.5 Investigation on the effects of various carbon sources on β-galactosidase expression

To investigate the effects of various carbon sources on the expression of β-galactosidases, glucose, fructose, galactose, arabinose, cellobiose, maltose, sucrose, raffinose (all from Sigma Aldrich, St. Louis, MO, USA) or lactose (Trade TCI Mark, Tokyo, Japan) at a 2% concentration was added into the MRS medium or MRS medium without glucose. Transformants and non-transformants were inoculated with these modified MRS media and incubated for 18 h at 37℃. β-galactosidase activities were assayed as described above.

3.2.6 Plasmid stability test

The stability of the plasmids constructed in this study was tested under non-selective conditions. Approximately 10^2 bacteria were inoculated in 8 ml of MRS and grown for 24 h. In these growth conditions, the number of bacteria in the culture reached approximately 5.0 x 10^8 to 2.0 x 10^9 CFU / ml, which corresponds to approximately 20 generations. The culture was diluted and inoculated in a new medium and cultured under the same conditions. After 100 generations, the plasmid stability was assessed by plating diluted aliquots on MRS agar medium with or without chloramphenicol and comparing colony counts on selective or non-selective MRS plates.

3.2.7 Hydrolysis of milk lactose by enzyme or transformants

Commercial milk from Seoul milk (Seoul dairy cooperative, Seoul, South Korea) was heated to 95℃ for 30 minutes for further sterilization and elimination of dissolved oxygen.

Then, 50 ㎍ of the crude enzyme extract produced by the method described above were added to 1 ml of the prepared milk. The mixtures were incubated for 8 h at 40℃, which is the optimal temperature for the hydrolytic activity of G1. After the incubation, each mixture was boiled at 100℃ for 5 min in order to inactivate the enzyme, and samples for HPLC were prepared as described by Marsili *et al.* (Marsili et al., 1981). Thus, 1 ml of water and 4 ml of acetonitrile were added to each 1 ml of sample and them shaken for 1 min and centrifuged to obtain a clear supernatant. The supernatant was filtered with a 0.2 ㎛, 13 mm PVDF Acrodisc syringe filter (Pall Corporation, Michigan, USA). Then, 5 ㎕ were injected for HPLC analysis.

5.0 x 10^8 CFU of RD47, BGN4, BGN4+pBES2 or BGN4+G1 were inoculated into 5 ml of the prepared milk. Incubation was carried out at 37℃, the optimum temperature for the bacterial growth, for 93 h. After the incubation, each mixture was boiled at 100℃ for 5 min in order to inactivate the bacteria, and samples for HPLC were prepared

as described above.

3.2.8 HPLC

The concentration of the lactose was determined by HPLC. The YL9100 HPLC system (Younglin, Gyeonggi-do, South Korea) was used, which was equipped with a YL9101 vacuum degasser, a YL9110 quaternary pump, a YL9131 column department, a 9170 RI detector, and a 9150 Autosampler.

An YMC-Pack Polyamine Ⅱ, 250 x 4.6 mm column (YMC, Gyeonggi-do, South Korea) was used with 75% acetonitrile as mobile phase at a flow rate of 1 ml / min. The column temperature was constant at 26℃. Calibration was carried out with external standards calculated by sugar solutions, for which the concentration is known. Samples were analyzed in a triplicate manner.

3.2.9 Statistical analysis of data

The results generated from this study were subjected to one-way analysis of variance (ANOVA) test at a 5% level of significance and post-hoc test (Duncan's multiple range test) using SigmaPlot 12 software.

3.3 Results

3.3.1 Gene cloning and heterologous expression of β-galactosidase from *B. longum* RD47

To obtain β-galactosidase over-expressing bifidobacteria using a heterologous gene from closely related organisms, it was necessary to find a gene exhibiting a strong β-galactosidase activity derived from bifidobacteria. In the preliminary studies, *Bifidobacterium longum* RD47 was one of the bifidobacterial strains with the greatest level of β-galactosidase activity (Han et al., 2014), and it was found that there were three β-galactosidase genes (G1, G3 and G4) in the full genome of RD47, which were cloned and characterized for hydrolysis and transgalactosylation, respectively. Among them, G1 showed the highest hydrolysis and transgalactosylation activity, and I chose it as the gene to be used in my experiment. The size of this gene is 3,072 bp, corresponding to 1,024 amino acids and a molecular mass of 112.64 kDa. The optimal pH and temperature for the hydrolytic activity of G1 are pH 8.5 and 40℃, respectively (Oh et al., 2017). *B. bifidum* BGN4 was the transformation host in which G1 was expressed heterologously. BGN4, which originated from the feces of a breast-fed infant, has been used as a probiotic strain in global food markets because of its various benefits. BGN4 exhibits a remarkable

colon cell binding ability (Kim et al., 2003; Ku et al., 2009), improves the immune function (Hong et al., 2009; Kim and Ji, 2006; Kim et al., 2007; Lee et al., 2002; Lee et al., 2006) and has anti-tumor effects (You et al., 2004). In addition to these features, BGN4 is a relatively genetically accessible strain among bifidobacteria and is therefore suitable for use as genetically engineered probiotics (Park et al., 2018).

G1 was amplified from the genome of RD47 and subcloned into pBES2 with combinations of several bifidobacterial promoters including its own promoter and transformed into BGN4 as the host. Information about the bifidobacterial promoters used in this study is listed in Table 3.2.

3.3.2 Comparison of hydrolytic activities between transformants and non-transformants

Strong promoters are required to promote the expression of a target gene. In previous studies, the strengths of various bifidobacterial promoters were compared using glucosidase as a reporter gene in BGN4. Among them, P504, P572 and P919 were relatively strong and constitutive (Wang et al., 2012; Youn et al., 2012). P504, P572 and P919 originated from *B. pseudocatenulatum* SJ32, *Bifidobacterium animalis* subsp. *lactis* SH5 and *B. bifidum*

BGN4, respectively. To develop bifidobacteria that strongly express β-galactosidase, I decided to use these promoters. G1 was expressed in BGN4 with combinations of P504, P572, P919 or its own promoter. The β-galactosidase activities of the recombinant bifidobacteria were determined using ρNPG as the substrate to compare the strengths of the promoters. As a result, BGN4+G1, which is BGN4 in which G1 is expressed with the combination of its own promoter, exhibited the highest hydrolytic activity (Fig. 3.1). When the enzymatic activities of the cultures incubated for 18 h were compared, the β-galactosidase activity of BGN4+G1 was approximately 4 times higher than that of wild-type BGN4 and 6 times higher than that of wild-type RD47, the original host of G1 (Fig. 3.1.A). The strength of the promoters was in the order of the G1 promoter followed by P572, P919 and P504 at 18 h incubation. However, when the incubation time was varied from 12 h to 30 h, the order of the other promoter strengths, except for the G1 promoter, was different according to the incubation time (Fig. 3.1.B). BGN4+G1 exhibited the highest hydrolytic activity regardless of the incubation time, for which the enzyme activity was 2.5 to 4.2 times higher than that of BGN4. Many previous studies reported that the consensus sequences (−35 and −10 regions) (Li and Zhang, 2014; McCracken et al., 2000), spacer sequences (Jensen and Hammer, 1998; Li and Zhang, 2014), TG motif (Helmann, 1995), UP elements (Estrem et al., 1998) and the promoter' s structural properties (Kumar and

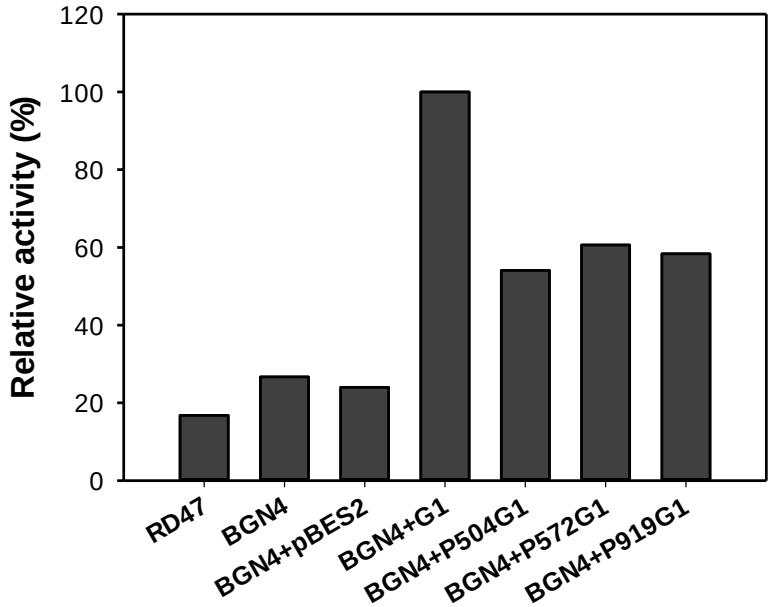
Bansal, 2018) affect the promoter strength. The promoters used in this study seem to differ in strength due to differences in those factors.

I next compared the enzymatic activities of the recombinants with 35 wild-type lactic acid bacteria present in nature including *Bifidobacterium*, *Lactobacillus*, *Streptococcus*, *Leuconostoc* and *Lactococcus* (Fig. 3.2). Enzyme activities of the recombinants were markedly superior to that of any other lactic acid bacteria used in this study. Incidentally, as already known, *Bifidobacterium* overall had a higher β-galactosidase activity than any other genera of lactic acid bacteria.

In a previous study, G1 was ligated into the pColdI Vector system (TAKARA) and expressed in *E. coli* BL21 (Oh et al., 2017). When the OD_{600} of the recombinant *E. coli* reached 0.4 to 0.6, the expression of the cloned gene was induced by adding 1 mM isopropyl 1-thio-β-D-galactopyranoside (Tokyo Chemical Industry Co., Ltd., Japan). The recombinant *E. coli* was then cultured aerobically at 37℃ for 21 h, and the enzyme activity of G1 was the highest at this point. At the time when the enzyme activity was the highest, the volumetric activity of the G1 recombinant *E. coli* was 0.46 U/ml fermentation broth and that of the BGN4+G1 was 0.85 U/ml fermentation broth, for which the G1 expression level of the recombinant bifidobacteria was approximately 2 times higher than

that of the recombinant *E. coli*. The utilization of BGN4+G1 is expected to be high because BGN4+G1 exhibited a better enzymatic activity than any other lactic acid bacteria and the G1 recombinant *E. coli* using an overexpression vector.

A


Relative activity (%)
120
100
80
60
40
20
0
RD47
BGN4
BGN4+pBES2
BGN4+G1
BGN4+P504G1
BGN4+P572G1
BGN4+P919G1

B

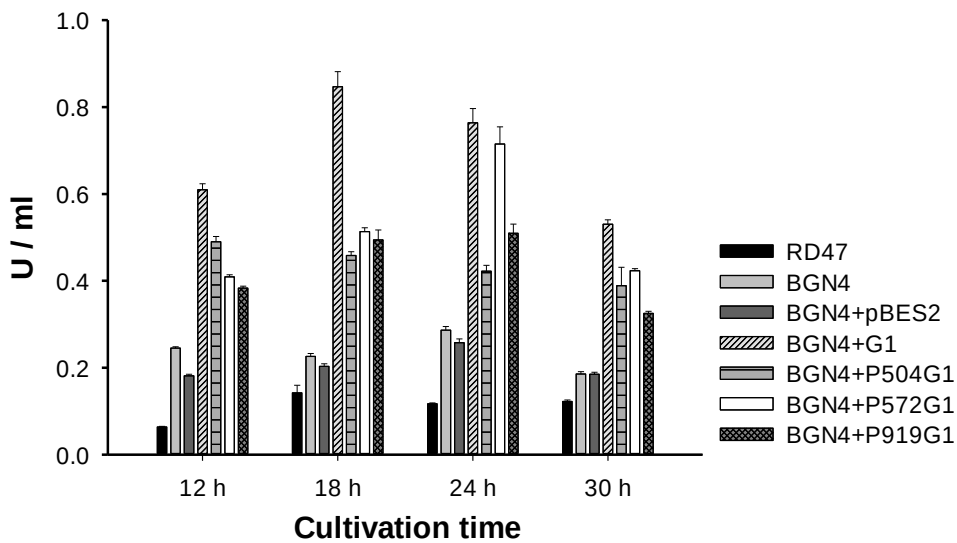

U / ml
1.0
0.8
0.6
0.4
0.2
0.0
12 h
18 h
24 h
30 h
Cultivation time
RD47
BGN4
BGN4+pBES2
BGN4+G1
BGN4+P504G1
BGN4+P572G1
BGN4+P919G1

Fig. 3.1 Comparison of the β-galactosidase activities between the wild-type *Bifidobacterium* sp. and recombinant BGN4 with various vector constructs

The bacteria were cultured at 37℃ for 12 to 30 h. (A) shows the relative activities at 18 h, and (B) shows the volumetric activities at incubation times from 12 to 30 h. The β-galactosidase activity was determined at 37℃ in 50 mM phosphate buffer (pH 7.0) with ρNPG as the substrate. RD47, *B. longum* RD47; BGN4, *B. bifidum* BGN4; others, *B. bifidum* BGN4 harboring each vector.

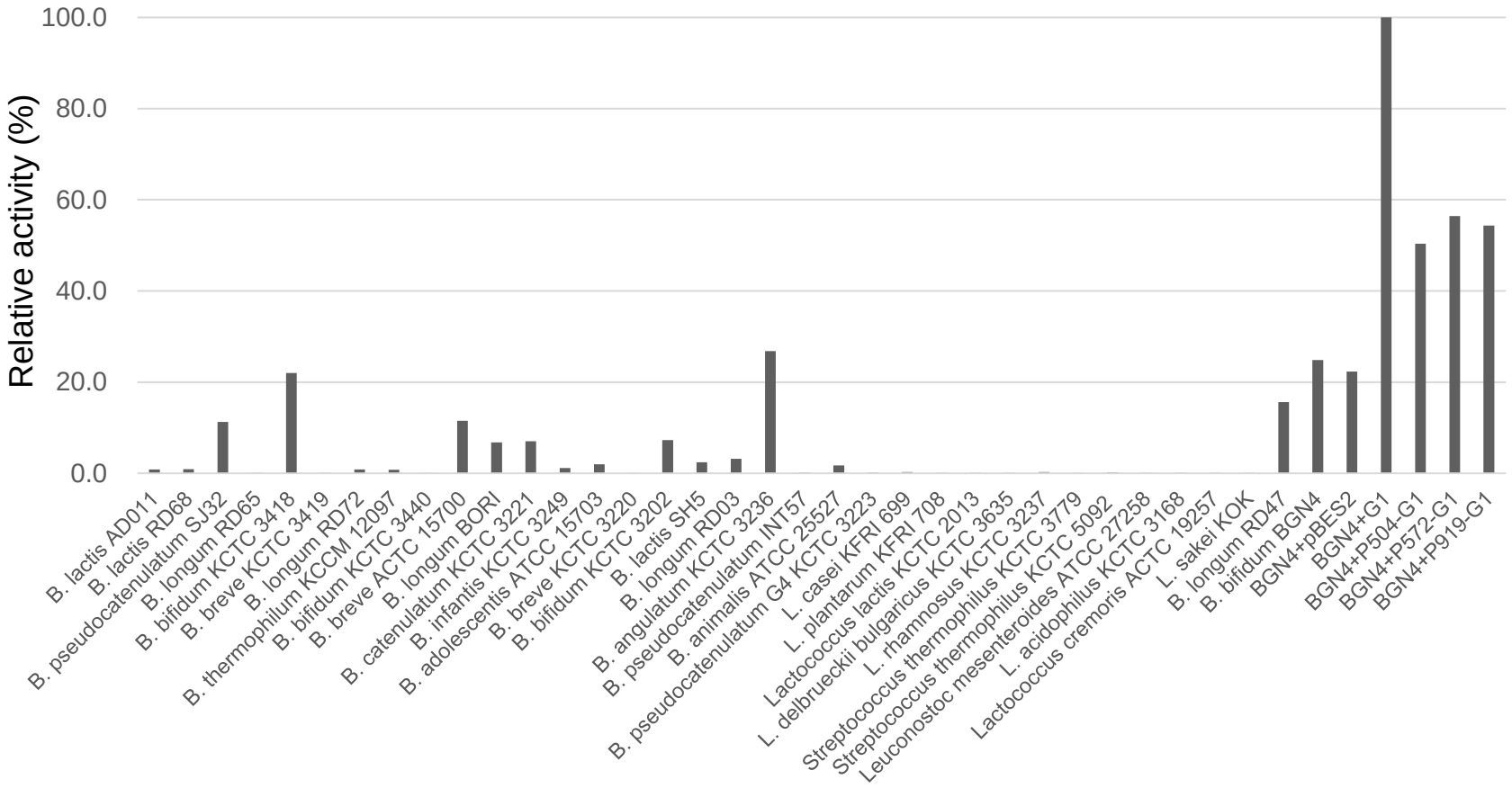

Relative activity (%)
100.0
80.0
60.0
40.0
20.0
0.0
B. lactis AD011
B. lactis RD68
B. pseudocatenulatum SJ32
B. longum RD65
B. bifidum KCTC 3418
B. breve KCTC 3419
B. longum RD72
B. thermophilum KCCM 12097
B. bifidum KCTC 3440
B. breve ACTC 15700
B. longum BORI
B. catenulatum KCTC 3221
B. infantis KCTC 3249
B. adolescentis ATCC 15703
B. breve KCTC 3220
B. bifidum KCTC 3202
B. lactis SH5
B. longum RD03
B. angulatum KCTC 3236
B. pseudocatenulatum INT57
B. animalis ATCC 25527
B. pseudocatenulatum G4 KCTC 3223
L. casei KFRI 699
L. plantarum KFRI 708
Lactococcus lactis KCTC 2013
L. delbrueckii bulgaricus KCTC 3635
L. rhamnosus KCTC 3237
Streptococcus thermophilus KCTC 3779
Streptococcus thermophilus KCTC 5092
Leuconostoc mesenteroides ATCC 27258
L. acidophilus KCTC 3168
Lactococcus cremoris ACTC 19257
L. sakei KOK
B. longum RD47
B. bifidum BGN4
BGN4+pBES2
BGN4+G1
BGN4+P504-G1
BGN4+P572-G1
BGN4+P919-G1

Fig. 3.2 Comparison of the β-galactosidase activities between 35 lactic acid bacterial strains and the recombinant BGN4

The bacteria were cultured at 37℃ for 18 h. The β-galactosidase activity was determined at 37℃ in 50 mM phosphate buffer (pH 7.0) with ρNPG as the substrate.

3.3.3 Effects of carbon sources on β-galactosidase expression

The effects of various carbon sources, including glucose, arabinose, galactose, fructose, sucrose, lactose, maltose, cellobiose and raffinose, on the activities of β-galactosidase from G1 transformants are shown in Fig. 3.3.

Among all the glucose-free MRS media with various carbon sources added, β-galactosidase in transformants grown on lactose had the highest activity at 18 h of cultivation at 37℃ (Fig. 3.3). This result was compatible with the results of Hsu CA *et al.* (Hsu et al., 2006; Hsu et al., 2005), who reported that lactose is an efficient inducer for the synthesis of β-galactosidase. The complete genome sequence of BGN4, which is the transformation host used in this study, has been determined (Accession No. NC_017999.1). According to the annotated gene information in this whole genome sequence, the lac operon exists in BGN4, and it suggests that the expression of β-galactosidases in BGN4 increases when lactose is the only sugar source (Bidart et al., 2018). This might be one of the most common causes for the highest β-galactosidase activity of the transformants when lactose is used as a carbon source except for glucose. At this point, it was wondered if the increased β-galactosidase activity of the BGN4 transformants in the presence of lactose is completely due to the lac operon in the BGN4 and not G1.

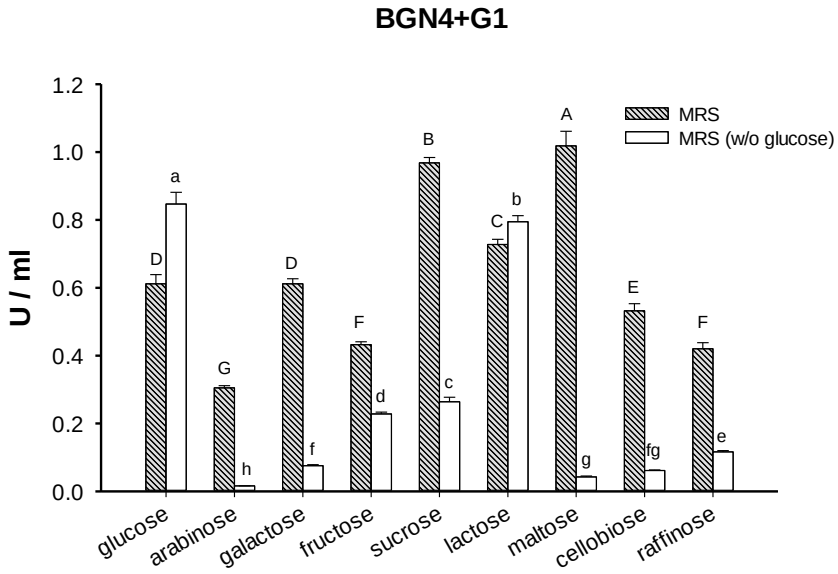
When the β-galactosidase activity of the wild-type BGN4 is subtracted from that of the G1 transformants, the enzyme activity of G1 alone can be obtained. The enzyme activity of G1 under lactose was at least 3 times more induced compared with the enzyme activity of G1 under other sugars used in this study except for glucose (data not shown). It means that not only the β-galactosidases of the BGN4 itself but also the heterologously expressed G1 was induced by lactose. Notably, BGN4+G1 and BGN4+P919G1 only exhibited such a phenomenon while the other transformants did not. Because the only difference between the former and latter is the promoter located upstream of G1, it seems that G1 itself does not react to lactose but rather that the promoter is responding. Because G1 is a β-galactosidase gene, it is reasonable that the G1 promoter is induced by lactose. However, P919 is a promoter for the ribosomal protein gene (Wang et al., 2012), and it is not understood why it reacts to lactose; thus, further study is needed. Among all the promoters used in the experiment, the G1 promoter was the most strongly induced under lactose based on the enzyme activity of G1.

In general, when the various carbon sources were added to the MRS medium containing glucose, the β-galactosidase activity tended to be greatly higher than that when the various carbon sources were added to the MRS medium without glucose. (Fig. 3.3) The case of BGN4+G1 under lactose was exceptional because the G1 promoter reacted strongly to lactose (Fig. 3.3.A). However, when 2% glucose

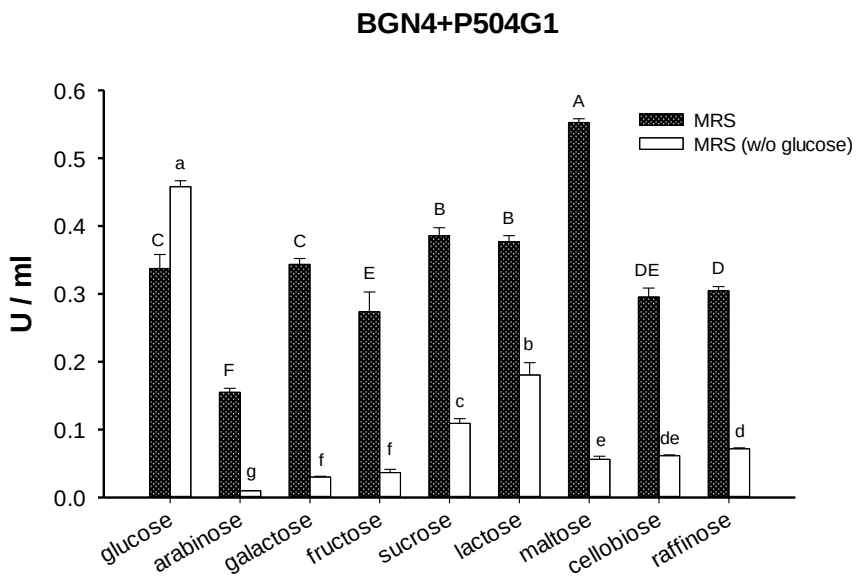
was added to the MRS medium containing glucose to yield a total of 4% glucose, the enzyme activity was lower than that when 2% glucose was added to the glucose-free MRS, which was consistent with previous studies showing that glucose at a high concentration inhibited β-galactosidase expression (Bidart et al., 2018; Chan et al., 2002; Han et al., 2014; Ilyés et al., 2004; Inchaurrondo et al., 1998). This phenomenon is known as carbon catabolite repression. In bacteria, the phosphotransferase system (PTS) (Bidart et al., 2018; Chan et al., 2002; Ullmann, 1996) and catabolite repressor/activator (Cra) model (Saier Jr and Ramseier, 1996) have been identified as mechanisms of carbon catabolite repression. Glucose is transported into the cell by PTS, which controls the cytoplasmic cAMP concentrations. Because cAMP-CAP complexes are required for the transcription of necessary promoters, cAMP control by the activities of PTS regulates the expression of genes related to the utilization of alternative carbon sources (Chan et al., 2002). On the other side, the Cra protein, either a repressor or activator of gene transcription by interacting with the RNA polymerase binding site, binds to the catabolites of glycolytic substrates, and these complexes are unable to bind to the DNA associated with the RNA polymerase binding site resulting in the catabolite repression effect (Saier Jr and Ramseier, 1996). A high glucose concentration seems to lower the activity of β-galactosidase by these mechanisms.

In all the transformants except for BGN4+P572G1, when 2% maltose was added to the MRS medium with glucose, the biosynthesis of β-galactosidase was highly induced compared to when glucose alone was used as a carbon source. On the other hand, BGN4+P572G1 had the highest enzymatic activity under the addition of 2% sucrose. These results were in contradiction to the results that when maltose or sucrose was used as the only carbon source, β-galactosidase was not induced much (Fig. 3.3). Presumably, sucrose and maltose are synergistic in beta-gal expression when glucose is present.

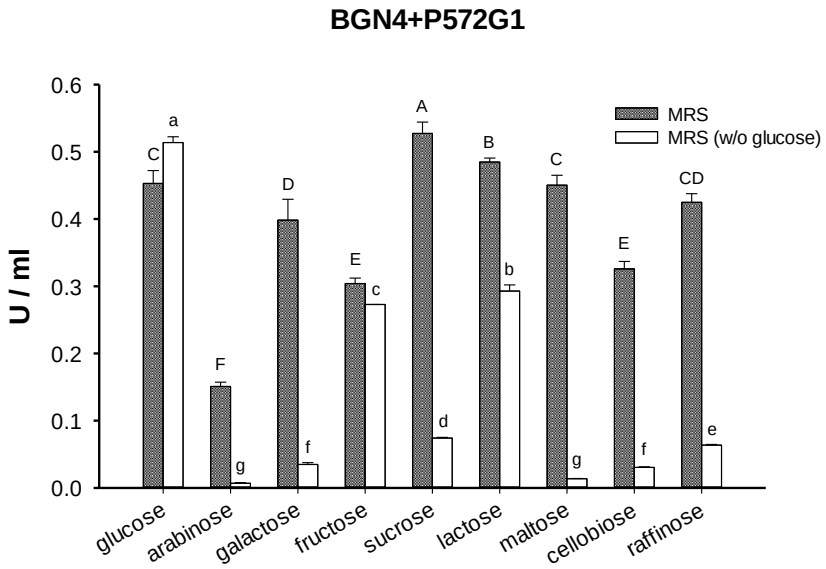
A


BGN4+G1
U / ml
1.2
1.0
0.8
0.6
0.4
0.2
0.0
MRS
MRS (w/o glucose)
D
a
G
h
D
f
F
d
B
c
C
b
A
g
E
fg
F
e
glucose
arabinose
galactose
fructose
sucrose
lactose
maltose
cellobiose
raffinose

B


BGN4+P504G1
U / ml
0.6
0.5
0.4
0.3
0.2
0.1
0.0
MRS
MRS (w/o glucose)
C
a
F
g
C
f
E
f
B
c
B
b
A
e
DE
de
D
d
glucose
arabinose
galactose
fructose
sucrose
lactose
maltose
cellobiose
raffinose

C


BGN4+P572G1
U / ml
0.6
0.5
0.4
0.3
0.2
0.1
0.0
MRS
MRS (w/o glucose)
C
a
F
g
D
f
E
c
A
d
B
b
C
g
E
f
CD
e
glucose
arabinose
galactose
fructose
sucrose
lactose
maltose
cellobiose
raffinose

D

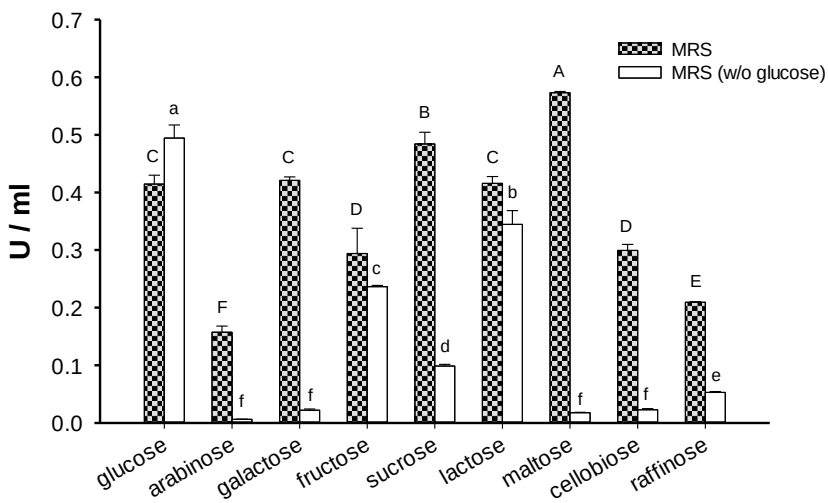

BGN4+P919G1
U / ml
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
MRS
MRS (w/o glucose)
C
a
F
f
C
f
D
c
B
d
C
b
A
f
D
f
E
e
glucose
arabinose
galactose
fructose
sucrose
lactose
maltose
cellobiose
raffinose

Fig. 3.3 Effects of the carbon sources on the production of β-galactosidase in the recombinant BGN4

The bacteria were cultured at 37℃ for 18 h, and the β-galactosidase activity was determined at 37℃ in 50 mM phosphate buffer (pH 7.0) with ρNPG as the substrate. ANOVA analysis was done independently for the results from the MRS and the results from the MRS without glucose. For the results from the MRS, statistically significant differences by Duncan's multiple range test ($p < 0.05$) were indicated by upper case alphabets, and for the results from the MRS without glucose, these were indicated by lower case alphabets.

3.3.4 Effects of non-selective conditions on β-galactosidase expression

The experiments from here on were conducted for BGN4+G1 because it exhibited the greatest β-galactosidase activity among all the G1 recombinants. The β-galactosidase expression of the G1 recombinant under non-selective conditions was next investigated. The BGN4+G1 recombinant was inoculated with MRS medium in the absence of antibiotics and incubated up to 60 h at 37℃. Up until the incubation time reached 60 h, the β-galactosidase activity of BGN4+G1 did not decrease in the absence of selection pressure; however, at most points, it was rather slightly higher than when antibiotics were present in the medium (Fig. 3.4).

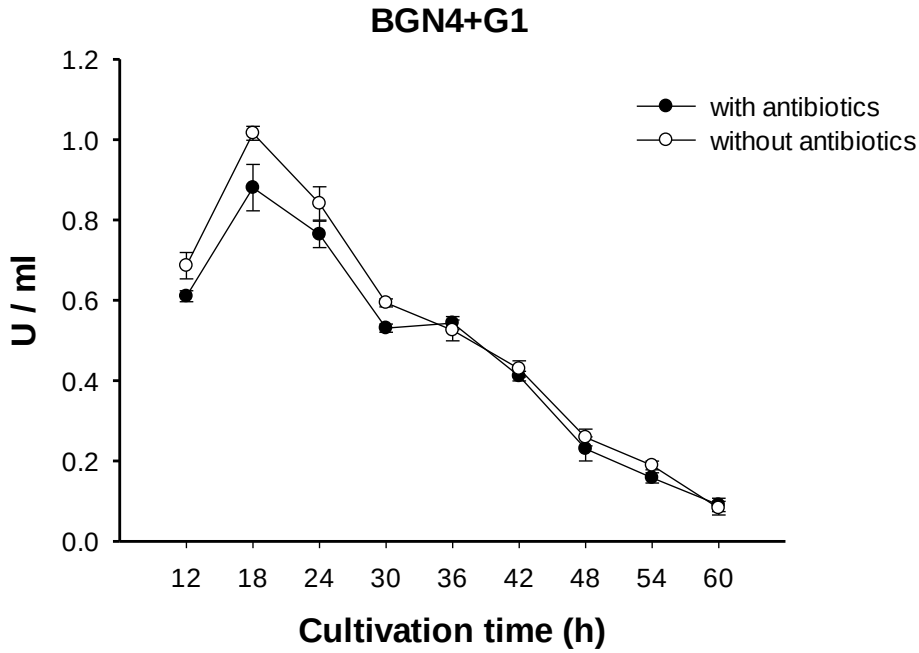


Fig. 3.4 Effects of non-selective conditions on β-galactosidase expression from the recombinant bifidobacteria

BGN4+G1 was cultured at 37℃ up to 60 h in MRS medium with or without chloramphenicol. The β-galactosidase activity was determined at 37℃ in 50 mM phosphate buffer (pH 7.0) with ρNPG as the substrate.

3.3.5 Plasmid stability under non-selective conditions

In order to estimate the stability of pB-G1, a pBES2 derivate containing the PG1-G1 expression cassette in BGN4, the strain was grown in MRS broth without antibiotics. After 100 generations, culture aliquots were plated on MRS agar with or without antibiotics (Gory et al., 2001). The presence of pB-G1 in the BGN4 colonies under the non-selective conditions was identified by comparing the restriction patterns with the original plasmid DNA. As a result, the number of transformants under nonselective conditions was maintained at 100% until 60 generations and thereafter, rapidly decreased and finally decreased by 53% after 100 generations compared with the selective condition. However, it was maintained consistently above a high bacterial count of 5.0×10^8 CFU/ml for the entire 100 generations.

For engineered bacteria, heterologous expression adds an unnatural load, which can challenge host cells with a significant burden (Ceroni et al., 2015; Ceroni et al., 2018; Silva et al., 2012). This metabolic burden can result in a decline of the biomass yield, cellular viability, recombinant protein productivity and plasmid stability (Carneiro et al., 2013). As bacterial generations proceed, the segregational instability of the plasmid appears to have a greater impact on the process productivity than on the structural stability, especially at higher frequencies under non-selective conditions,

and the formation and accumulation of a plasmid-free population reduces the final plasmid DNA yields (Oliveira et al., 2009). According to the results in this study, in the absence of antibiotics, the stability of pB-G1 was maintained at 100% until 60 generations, the bacterial counts for the BGN4+G1 transformant were kept above 5.0 x 10^8 CFU / ml for 100 generations, and the enzyme activity did not decrease for 60 h compared with the selective conditions. It is believed that the stability of the subcloned plasmid DNA and the recombinant protein expression will promote the use of the BGN4+G1 transformant and pB-G1 plasmid in industry or further laboratory studies.

3.3.6 Lactose hydrolysis

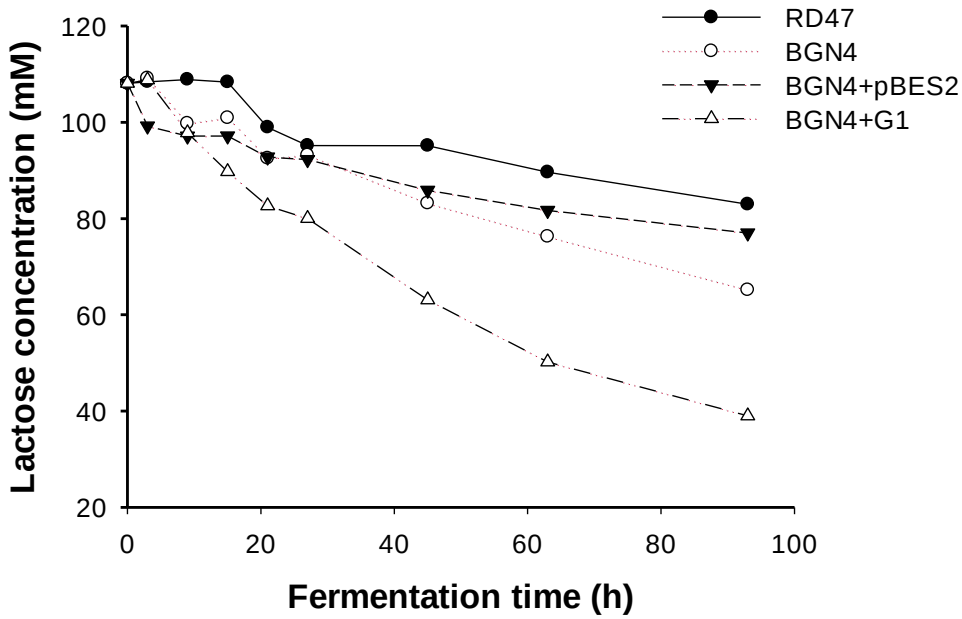
Lactose hydrolysis experiments were carried out with BGN4+G1, which has the highest enzyme activity among the recombinant bifidobacteria, BGN4, BGN4 containing pBES2 (BGN4+pBES2), which is the *Bifidobacterium-E. coli* shuttle vector used for the G1 subcloning, and RD47 by reacting them with milk and by reacting their crude enzyme extracts with milk. The lactose hydrolysis capacity in milk was determined by measuring the residual lactose in the milk by HPLC.

After inoculating 5.0 x 10^8 CFU of each bacteria into 5 ml of milk, the amount of residual lactose in the milk was measured for 0 – 93 h, and the result is shown in Fig. 5A. BGN4+G1 consumed the most lactose in the milk within the same time period, followed by BGN4 and RD47. This is consistent with the order of β-galactosidase activity in each bacteria (Fig. 1). It was reported that only about 30% of the lactose is removed from fermented milk products (Harju et al., 2012), and in reality, BGN4 and RD47 eliminated only 17% and 35% of the lactose in the milk at 93 h, respectively. However, BGN4+G1 removed 50% of the lactose in the milk already at 63 h and finally 61% at 93 h. This is about twice the lactose removal rate of conventional fermented milk products.

After inoculating 50μg of protein as a crude enzyme extract from each bacteria into 1 ml of milk, the amount of residual lactose in the milk was measured up to 8 h, and the result is presented in Fig. 5B. The β-galactosidase of BGN4+G1 consumed the most lactose in the milk within the same time period followed by that of RD47 and BGN4. This order is consistent with the order for the volumetric activity in the crude enzyme extract of each bacteria. The volumetric activities of β-galactosidase in 50 μg of protein for RD47, BGN4, BGN4+pBES2 and BGN4+G1 were 0.087, 0.053, 0.041 and 0.166 U/ml, respectively. The β-galactosidase of BGN4+G1 hydrolyzed 51% of the lactose in the milk at 2 h, but after that, the enzyme

activity sharply decreased, and the lactose in the milk was slowly decomposed and only 60% was hydrolyzed at 8 h.

A


RD47
BGN4
BGN4+pBES2
BGN4+G1
Lactose concentration (mM)
120
100
80
60
40
20
0
20
40
60
80
100
Fermentation time (h)

B

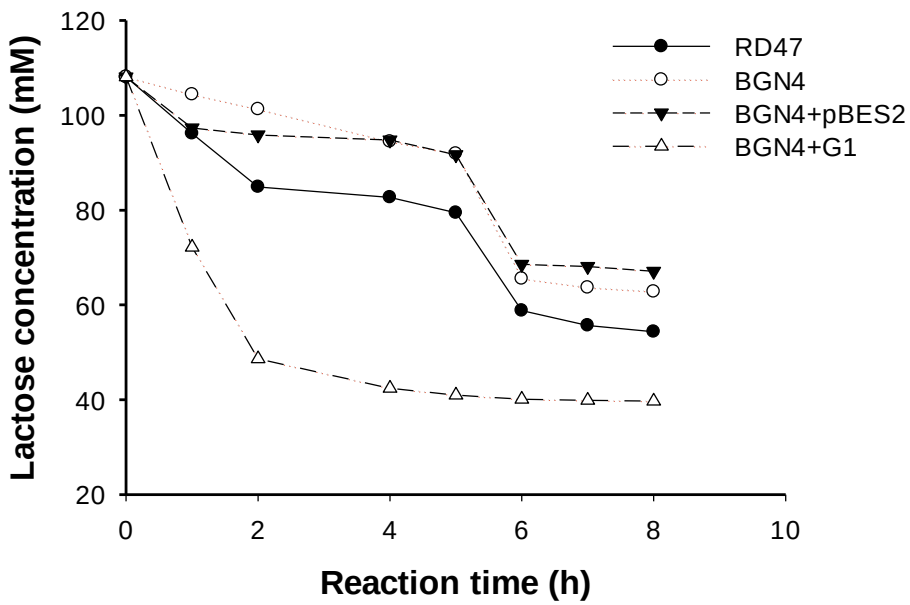

RD47
BGN4
BGN4+pBES2
BGN4+G1
Lactose concentration (mM)
120
100
80
60
40
20
0
2
4
6
8
10
Reaction time (h)

Fig. 3.5 Lactose hydrolysis in milk by bacteria or β-galactosidase

After reacting the milk and bacteria at 37℃ (A) or the milk and β-galactosidase of the crude enzyme extract at 40℃ (B) for a certain period of time, residual lactose was measured by HPLC. RD47, *B. longum* RD47; BGN4, *B. bifidum* BGN4; others, *B. bifidum* BGN4 harboring each vector

3.4 Discussion

In this study, a β-galactosidase over-expressing bifidobacteria strain was developed by using a heterologous gene from *B. longum* RD47 which is one of the bifidobacterial strains with the greatest level of β-galactosidase activity. Among the recombinant bifidobacteria, BGN4+G1 has the highest β-galactosidase activity, for which the hydrolytic activity was 2.5 to 4.2 times higher than that of BGN4 and 4.3 to 9.6 times higher than that of the wild-type RD47 (Fig. 1B). The β-galactosidase level of BGN4+G1 was remarkably superior to that of any of the other 35 lactic acid bacteria used in this study (Fig. 2). Furthermore, at the time when the enzyme activity was the highest, the volumetric activity of BGN4+G1 was 0.85 U/ml fermentation broth, which was twice as high as that of the G1 recombinant *E. coli* using an overexpression vector. In this regard, industrial or research utilization of BGN4 + G1 is expected to be high.

It has been generally reported that only about 30% of the lactose is removed from conventional fermented milk products (Harju et al., 2012; Ohlsson et al., 2017), and in agreement with this, BGN4, which has the second highest β-galactosidase activity among the 35 lactic acid bacteria present in nature, only eliminated 35% of lactose in milk. Because lactose intolerant subjects will have a problem with these

products, when making fermented milk, fermentation by a starter and the reaction with β-galactosidase sometimes proceed together (Dekker et al., 2019). However, BGN4+G1 solely hydrolyzed 50% of the lactose in milk at 63 h and finally 61% at 93 h during fermentation (Fig. 5A). This suggests that it is necessary to improve the β-galactosidase activity of the starter strains by expressing an exogenous enzyme or by mutation in order to reduce the residual lactose content of fermented milk for lactose intolerant subjects. From my results, the G1 expression cassette used to construct BGN4+G1 can be used to increase the β-galactosidase activity of starter strains using a food-grade vector or CRISPR technology because of the safety of the genetic origin and the strong intensity of the gene expression.

Currently, in the production of lactose-free milk, β-galactosidase is mainly used, and the process is carried out only in such a way that a soluble enzyme is added without using the enzyme-immobilization method due to problems with the microbial stability of the final product (Dekker et al., 2019). Although BGN4+G1 does not have a higher enzyme activity than other recombinant strains (Becerra et al., 2001; Ding et al., 2018; Yuan et al., 2008), it has an advantage in that it is safe because the bacterial host and gene are derived from bifidobacteria, the GRAS bacteria, and the β-galactosidase activity is remarkably high compared with lactic acid bacteria in nature. Furthermore, the β-galactosidase of BGN4+G1 hydrolyzed half of

the lactose in whole milk in 2 h using a crude enzyme extract with 50 μg of protein (Fig. 5B), and it is expected to have a greater effect if the amount of crude enzyme extract to be used for the reaction is increased. The simple purification of β-galactosidase from BGN4-G1 by using a his-tag will have industrial significance, such as using it in the production of lactose-free milk or fermented milk

Chapter 4.

Lactose intolerance alleviation in post−weaning Balb/c mice by feeding β−galactosidase high expressing *Bifidobacterium bifidum*

4.1 Introduction

Lactose intolerance (LI) is a condition which features the inability to digest lactose due to a deficiency of β-galactosidase (lactase) in the digestive tract (Li et al., 2012; Oak and Jha, 2018). Lactase deficiency causes lactose to pass into the colon because it is not digested and absorbed in the small intestine. Then, lactose is converted into short chain fatty acids and gas (methane, hydrogen and carbon dioxide) by colonic microflora (Vonk et al., 2012), and elevated osmotic pressure caused by the lactose itself and the lactose fermentation products increase the intestinal water content. These phenomena lead to typical clinical symptoms including diarrhea, bloating, abdominal pain, nausea, bloating, flatulence, blanching and cramps between 30 min and 2 h after lactose intake (Harrington and Mayberry, 2008; Nivetha and Mohanasrinivasan, 2017). Lactose intolerant people have poor quality of life activities because of the acute clinical symptoms after the intake of lactose-containing foods such as milk and milk products (He and Yang, 1999). About 75% of the world's adult population undergo LI, and the prevalence of LI varies by race. It has been reported that 5 - 15% of Europeans and North Americans, 50 - 80% of Hispanic people, 60 - 80% of African-Americans and Ashkenazi Jewish people and almost 100% of Asian and American Indian people have a lactase deficiency

(Heyman, 2006; Misselwitz et al., 2013). Therefore, LI is a worldwide issue in terms of public health management, especially in some regions including Asia (Li et al., 2012).

A common treatment for managing LI tends to exclude milk from the diet. However, this approach may have serious nutritional disadvantages mainly reducing the intake of calcium, phosphorus and vitamins (Montalto et al., 2006). These nutritional limits can lead to a lower bone density (Di Stefano et al., 2002) and a significant risk of osteoporosis (Savaiano, 2011). Fermented milk is usually thought to be an ideal milk substitute for LI subjects, but it was reported that only about 30% of the lactose is removed from fermented milk products (Harju et al., 2012). To overcome the limits of dietary approaches, several studies have been conducted to find alternative strategies, such as using exogenous β-galactosidase, yogurt and probiotics for their bacterial β-galactosidase activity (Montalto et al., 2006). Among them, probiotics, particularly strains belonging to *Bifidobacterium* and *Lactobacillus*, have recently attracted attention as a potential treatment for lactase deficiency (Oak and Jha, 2018). However, contradictory results have been reported in previous studies in which probiotics were effective for LI (Almeida et al., 2012; He et al., 2008; Hiele et al., 1988; Rabot et al., 2010) or not (Kim and Gilliland, 1983; Levri et al., 2005; Park et al., 1999a; Saltzman et al., 1999). Concerning this phenomena, Kara M. Levri *et al.* (Levri et al., 2005) suggested that specific probiotic strains seem to be

effective for LI, so further studies on specific strains are necessary to reveal this potential therapeutic relationship.

Recently, a mouse experiment was performed to investigate the alleviation of LI by *Lactococcus lactis* expressing heterologous β-galactosidase (Li et al., 2012). This study reported that the strain used showed the effect of alleviating LI symptoms, and the probable mechanism for it was the predominant colonization of lactic acid bacteria, mainly *Bifidobacterium.* I hypothesized that *Bifidobacterium* may be effective in alleviating LI, but the reason for the contradictory results of previous studies is that there is a difference in the β-galactosidase activity between *Bifidobacterium* strains used in the experiments. To prove the hypothesis, the aim of this study was to verify the effect of the β-galactosidase activity of bifidobacteria on LI alleviation by administering β-galactosidase reduced bifidobacteria, wild-type bifidobacteria and β-galactosidase over-expressing bifidobacteria to post-weaning Balb/c mice which have been shown to be the LI model mice (He et al., 2005).

4.2 Materials and Methods

4.2.1 Randomized mutagenesis by MNNG

Randomized mutagenesis by MNNG (1-methyl-3-nitro-1-nitrosoguanidine), a chemical mutagen, was performed as described by S. A. Ibrahim *et al.* (Ibrahim and O'Sullivan, 2000) with several modifications. Overnight cultures of *B. bifidum* BGN4 were diluted into 8 ml of fresh MRS broth supplemented with 0.05% L-cysteine·HCl and incubated until the OD_{600} reached 0.4 (early-exponential phase). After centrifugation, the pellet was washed in 1 ml of 100 mM citrate buffer (pH 5.5). The suspended cells were mixed with 1 ml of the same buffer as above containing 5 mg/ml of MNNG and incubated at 25℃ with gentle agitation. After 30 min of incubation with a mutagen, the mixture was centrifuged and washed twice with 1 ml of 100 mM phosphate buffer (pH 7.0). The mutagenized cultures were then plated on X-gal based MRS medium. All plates were incubated at 37℃ for at least 72 h. Colonies that exhibited a relatively white color were selected to assay quantitatively the β-galactosidase activities using para-nitrophenyl (pNP) β-D-galactopyranoside.

4.2.2 Assay for β-galactosidase activity

The β-galactosidase activity was measured by the release of para-nitrophenol from para-nitrophenyl (pNP) β-D-galactopyranoside (Sigma Aldrich, St. Louis, MO, USA). To perform this assay, first, a crude enzyme extract of bacteria was prepared as follows. The incubated bacteria were centrifuged (10,000 ×g for 3 min at 4℃), and the harvested pellet was washed with 50 mM sodium phosphate buffer (pH 7.0). The washed bifidobacterial cells were resuspended in the same buffer and disrupted with a sonicator (Q500; K-corporation, Gyeonggi-do, South Korea) for 1.0 s with 1.0 s off intervals for 15 min. The disrupted bacterial solution was centrifuged at 10,000 ×g for 10 min at 4℃, and the supernatant was used as crude enzyme extract to assay the β-galactosidase activity. Thus, 80 μℓ of the crude enzyme extract were added to 20 μℓ of 5 mM pNP β-D-galactopyranoside suspended in 50 mM of a sodium phosphate buffer (pH 7.0). The mixture was incubated at 37℃, and the reaction was stopped by adding 100 μℓ of 1 M Na_2CO_3. The activity was measured in a 96-well microplate at 405 nm using a spectrophotometer (Model 680 Microplate reader; Bio-Rad, Hercules, CA, USA). One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of pNP per minute at 37℃ and pH 7.0. The volumetric activity (enzyme units per ml of fermentation broth) was determined as the units of β-galactosidase activity.

4.2.3 Bacterial binding to the Caco-2 cell line

The colonocyte-like cell line Caco-2 was used to investigate the adhesion ability of the wild-type BGN4 and mutant BGN4 to the mucosal surfaces in the intestine. Caco-2 cells were purchased from the Korean Cell Line Bank (KCLB, Seoul, South Korea). The culture and maintenance of the cells were carried out following standard procedures (Sánchez et al., 2010) using DMEM medium (Gibco Invitrogen, Paisley, UK) containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotic antimycotic solution.

For the adhesion assays, cells were seeded at a concentration of 5.0 X 10^3 cells/well in 24-well plates until a confluent differentiated state was reached, that is, after 14 days in culture and complete differentiation. The cell culture medium was changed every 2 days and replaced with antibiotic-free DMEM medium 24 h before the adhesion test. The viable cell number was about 1.08 X 10^5 cells per well.

Bifidobacteria were incubated for 18 h and washed twice with PBS buffer (pH 7.4) and then resuspended in antibiotic-free DMEM medium at a concentration of 10^9 CFU / ml. Caco-2 cellular monolayers were also gently washed with PBS buffer (pH 7.4), and bacterial suspensions were added to a final count of 10^9 CFU. Adhesion experiments were carried out for 1 h at 37℃ in a humidified atmosphere of 5% CO_2, and afterwards, the wells were

carefully washed to remove any unattached bacteria. After detachment with 0.25% Trypsin-EDTA solution (Sigma, St. Louis, MO, USA), the cells were transferred to a 1.5 ml tube. The wells were rinsed with 200 ㎕ PBS buffer (pH 7.4) which was also transferred to the 1.5 ml tube above. The bacteria attached to the Caco-2 cells were quantified by real-time PCR.

4.2.4 Experimental animals

All animal study designs and procedures were approved by the local animal ethics committee at the Seoul National University (approval number : SNU-180917-1-1). Animals were maintained and treated in accordance with the guideline of the Institute of Laboratory Animal Resource (Seoul National University, Seoul, South Korea). Post-weaning Balb/c male mice (9-10 weeks old) were used (DBL, Chungcheong-do, South Korea). They were maintained in a temperature-controlled environment (22 ± 2℃) at a relative humidity of 50 ± 10% and a 12 h light/dark cycle.

4.2.5 Animal groups and oral administration of bacteria

Balb/c mice were acclimatized for a week and randomly divided into seven groups (n=14) based on their body weight. They were fed a commercial diet, AIN-93G (DYbiotech, Seoul, South Korea) and tap

water *ad libitum* during the experiment.

The bacterial intake groups were administered daily with 1×10^9 CFU of lyophilized *B. bifidum* BGN4, 1×10^9 CFU of lyophilized *B. bifidum* BGN4-pBES2, 1×10^9 CFU of lyophilized β-galactosidase over-expressing BGN4 (BGN4-G1) or 1×10^9 CFU of lyophilized β-galactosidase reduced BGN4 (BGN4-bgr) in a normal saline (NS) suspension with a total volume of 200 ㎕, respectively. All bacteria to be consumed by the mice were lyophilized from Bifido Inc., Gangwon-do, South Korea. For the untreated, lactase-treated and uninduced mice, 200 ㎕ of NS were ingested daily. In the case of the lactase-treated group, a commercial lactase tablet with an activity of 9,000 U/per one tablet dissolved in a NS suspension with a total volume of 200 ㎕ was administered shortly before lactose intolerance induction. The commercial lactase used in this experiment was Lactaid Fast Act (HP Hood LLC, Lynnfield, MA, USA). Because an adult dose of the lactase product is one tablet at a time, the dose of lactase for mice was determined as 4.6 mg/kg of body weight by converting it assuming that the average weight of an adult is 60 kg.

4.2.6 Sample collection

After daily administration for 2 weeks, all mice were fasted for 12

h before lactose challenge. The feces of seven mice from each group were collected for microbiome analysis and then challenged with lactose (25 g per 60 kg of body weight) to investigate intestinal motility. The other seven mice of each group were also challenged with the same concentration of lactose as above to investigate stool frequency and total feces weight within 6 h post-challenge. At this point, the fasting plasma glucose level, the baseline, was measured in the tail vein blood just before lactose administration and after the lactose challenge, and the plasma glucose was again obtained from the tail-vein blood 30 min later. After 6 h post-challenge, they were sacrificed, and 10 cm of the small intestine near the stomach and caecum were anatomized out to measure the intestinal β-galactosidase activity and SCFA concentrations, respectively.

4.2.7 Diarrhea index determination

The diarrhea index was measured by the stool frequencies, the total feces weight and the intestinal motility. To determine the diarrhea index, mice were fasted for 12 h before lactose challenge. The stool frequencies and the total feces weight of half of the mice in one group were recorded within 6 h post-challenge, and the intestinal motility was tested in the other half in one group. For the intestinal motility test, mice were orally administered with 0.2 ml of a charcoal suspension containing 10% gum Arabic (Sigma Aldrich, St. Louis, MO,

USA) and 5% activated charcoal (Samchun, Seoul, South Korea) 20 min after lactose challenge, and sacrificed 20 min after the administration of the charcoal suspension. Then, the small intestine was anatomized out from the pylorus to the ileocecal junction, and the distance from the pylorus to the front edge of the charcoal particle and the total length of intestine anatomized out were measured, respectively. The percentage of the running distance of the charcoal particle in total small intestinal length was calculated to express the intestinal motility.

4.2.8 Measurement of the β-galactosidase activity in the small intestine

The small intestine with ten times the volume of ice-cold 0.9% NS were homogenized with Tissue-Tearor (985370; BioSpec Products Inc., Bartlesville, OK, USA) for 1 min. Then, the cell and tissue debris were spun down at 1,000 ×g for 10 min, and the supernatant was collected to measure the β-galactosidase activity. The β-galactosidase activity of the supernatant was determined by the release of para-nitrophenol from para-nitrophenyl (pNP) β-D-galactopyranoside (Sigma Aldrich, St. Louis, MO, USA) as the substrate at 37℃ in phosphate buffer at pH 7.0. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated

1 μmol of pNP per minute at the given conditions.

4.2.9 SCFA analysis

The cecal samples (100 mg) were homogenized in 400 ㎕ of DW. Thereafter, these were acidified with 25% metaphosphoric acid (Sigma Aldrich, St. Louis, MO, USA) at a 1:5 ratio (1 volume of acid for 5 volumes of a sample) for 30 min on ice. Samples were then centrifuged at 15,000 x g for 15 min at 4℃, and the supernatant was stored at −80℃ until it was analyzed by HPLC.

The supernatant was injected into an YL9100 HPLC system equipped with an YL9170 RI detector and the Younglin Autochro-3000 data system software (Younglin, Gyeonggi-do, South Korea). SCFAs were separated on an Aminex HPX-87H Ion Exclusion column (300 X 7.8 mm, 9 ㎛, Bio-Rad, Hercules, CA, USA) which was run isocratic with 5 mM sulfuric acid at a flow rate of 0.6 ml per min at a temperature of 35℃.

4.2.10 Microbiome analysis by 16S metagenomic sequencing

4.2.10.1 DNA extraction from the sample collection

The fecal (180 – 220 mg) samples were collected from the mice of each group, stored at −80℃ until the genomic DNA was extracted and used for DNA extraction. The microbial genomic DNA in the stools was extracted using a QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) following an initial bead−beating step and with some modifications to increase the DNA concentration. Fecal samples were finely ground after lyophilization and transferred to a 2 ml tube containing sterile 0.5 mm zirconia beads (BioSpec Products Inc., Bartlesville, OK, USA). Then, 1.4 ml of ASL buffer (QIAGEN) were added to each tube, and the samples were homogenized by bead−beating with a tissue lyser (TissueLyser Ⅱ; QIAGEN) for 30 s at a speed of 30 frequency / s. The lysis temperature and time were increased to 95℃ and 10 min, respectively. The extracted DNA samples were stored at −20℃ until 16S metagenomic sequencing.

4.2.10.2 Library preparation

DNA concentration was measured using a Qubit® 3.0 Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and diluted to 5 ng / ㎕. The microbial genomic DNA (5 ng / ㎕) was amplified using the amplicon primers with overhang adapters attached targeting variable V3 and V4 region of the 16S rRNA gene. An Agencourt

AMPure XP beads (Beckman Coulter, Brea, CA, USA) were used to purify 16S V3 and V4 amplicon away from free primers and primer dimer species.

The 16S V3 and V4 amplicon was amplified with dual-index primers via PCR and purified with Agencourt AMPure XP beads. The index PCR amplicon possessed specific barcode sequences to distinguish among each other in the pooled library.

The concentrated library was measured using a Qubit® 3.0 Fluorometer (Thermo Fisher Scientific Inc.) and diluted using Resuspension Buffer (RSB; Illumina, San Diego, CA, USA) to 4 nM. 5 ㎕ aliquot of diluted DNA from each samples were mixed for pooling libraries.

4.2.10.3 Sequencing via an Illumina MiSeq platform

A pooled library was denatured with NaOH, diluted to 8 pM with hybridization buffer (HT1; Illumina). A PhiX control library (Illumina) was denatured with NaOH, diluted to 8 pM with HT1. The pooled library was mixed with the PhiX control (30%, v/v) and the combined library and PhiX control was loaded on a MiSeq® v2 (500 cycle) Reagent cartridge (Illumina) for sequencing. All sequencing procedures were monitored through the Illumina BaseSpace® website.

4.2.10.4 Sequencing data processing

Sequencing analysis, including demultiplexing and removal of indices, was performed using the bacterial metagenomics workflow in the MiSeq Reporter software (Illumina). The classification step uses a proprietary algorithm that provides each taxonomic level classification for paired-end reads.

4.2.11 Statistical analysis

In the graphs related to LI symptoms (Fig. 1 - Fig. 5), results were compared using an unpaired, one-tailed nonparametric *t* test using GraphPad Prism 8 software to compare each experimental group to untreated group. Significant *P* values for all comparisons are depicted in figures as follows: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

In the graphs related to microbiome and SCFA analysis (Fig. 6 - Fig. 9), results were compared using a nonparametric one-way ANOVA test (Kruskal-Wallis H) and post-hoc test (Dunn's multiple comparisons test) in GraphPad Prism 8 software. Significant *P* values for all comparisons are depicted in figures as follows: $*P < 0.05$ and $**P < 0.01$.

4.3 Results

4.3.1 Construction of the β-galactosidase reduced and over-expressing *B. bifidum* BGN4

To investigate the effect of the β-galactosidase activity of *Bifidobacterium* on LI, construction of the β-galactosidase reduced and over-expressing bifidobacteria by genetic modification was required. The bifidobacterial strain used in this study was selected as *B. bifidum* BGN4 because it is genetically accessible (Park et al., 2018) and has a remarkable colon cell adhesive ability among the other bifidobacteria (Kim et al., 2003; Ku et al., 2009). Previously, I constructed a recombinant BGN4 strain expressing heterologous β-galactosidase, BGN4-G1 (unpublished study). Because the β-galactosidase activity of BGN4-G1 was 2.5 to 4.2 times higher than that of the wild-type BGN4 and markedly superior to those of the 35 wild-type lactic acid bacteria, BGN4-G1 was chosen as the β-galactosidase over-expressing strain against the wild-type BGN4 in this study. Meanwhile, β-galactosidase reduced BGN4 was obtained by randomized mutagenesis of BGN4. Because BGN4 has six putative β-galactosidase genes in its whole genome (Accession No. NC_017999.1), it is somewhat unreasonable to obtain a β-galactosidase-deficient BGN4 strain through targeted mutagenesis.

Chemical mutagenesis for BGN4 was conducted as a reliable method to yield the lowest kill rate with an acceptable frequency of mutagenesis. After the mutagen treatment, 11 colonies were obtained, and additional validation experiments were performed. After investigating their enzymatic activities, growth rate and colon cell binding ability, a strain named BGN4-bgr was finally selected, whose β-galactosidase activity was significantly lower than that of BGN4 and its characteristics, which could inadvertently affect the results of this study, are similar to BGN4. The results of the comparison of the β-galactosidase activity, growth rate and colon cell adhesive ability between the wild-type BGN4 and the mutant are shown in Fig. 4.1. Based on the MRS medium, the growth curve of BGN4-bgr was similar to that of BGN4 in both cases when glucose (Fig. 4.1.A) or lactose (Fig. 4.1.B) was used as the sole carbon source. The growth rate of BGN4-bgr tended to be slower at the beginning of the incubation, rapidly recovered, and the maximum OD_{600} and the time to reach the value were similar in both BGN4 and the mutant. In the MRS medium when glucose was the carbon source, the β-galactosidase level of the BGN4-bgr was 142.8 times lower than that of BGN4 at 12 h of incubation, after which the enzyme activity slightly recovered but still 3.7 to 9.7 times lower during the incubation time (Fig. 4.1.A). When lactose was the carbon source, the β-galactosidase activity of the BGN4-bgr was 2.7 to 5 times lower than that of BGN4 depending on the incubation time (Fig. 4.1.B). Next, the

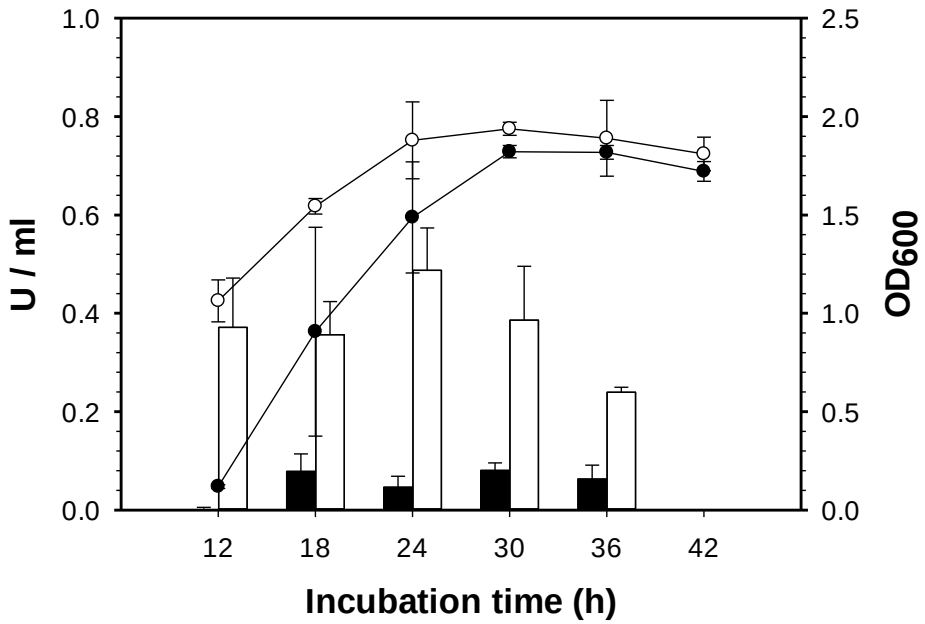
colon cell binding ability of BGN4 and BGN4-bgr was investigated. Because adherence on intestinal epithelial cells is an essential process for the colonization of microorganisms in the gut (Kadlec and Jakubec, 2014; Parvez et al., 2006), if there is a difference in the ability to adhere to the intestine between BGN4 and BGN4-bgr, the level of colonization in the gut will be different, which can act as a bias in this study. As a result, BGN4-bgr had a tendency to exhibit a better colon cell binding ability than BGN4, but there was no significant difference (Fig. 4.1.C). Whole genome sequencing of BGN4-bgr was performed to investigate whether mutations directly occurred in the β-galactosidase genes. Then, the genome of BGN4-bgr was compared with the putative β-galactosidase genes in BGN4 (Table 4.2). As a result, it was found that mutations occurred in two out of five genes. Both of them had a point mutation in the protein coding region rather than in the promoter region. These direct mutations of β-galactosidase genes in BGN4-bgr are considered to be a cause of lower β-galactosidase activity of BGN4-bgr than BGN4.

As mentioned above, the β-galactosidase reduced BGN4 was developed through randomized mutagenesis, so the mutant has the limitation that mutations may have occurred not only in genes associated with β-galactosidase expression but also in unexpected genes that inadvertently affect the results of my experiments. To overcome this limitation, I additionally confirmed several factors that

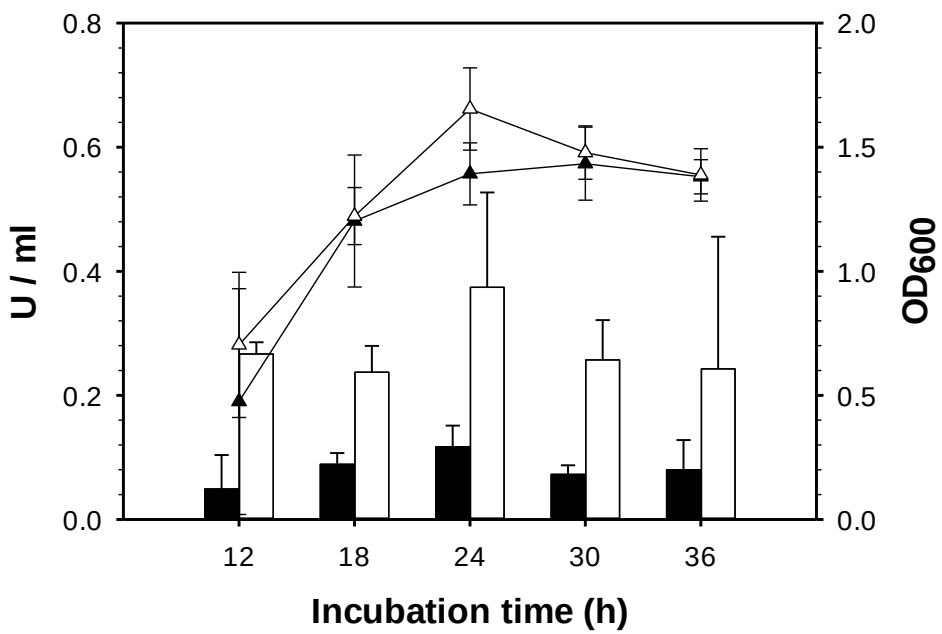
might influence this study, such as growth rates and the ability to adhere to the intestine, and finally selected the mutant.

Finally, when the lyophilized bacterial powder for administration to mice was resuspended in phosphate buffer (pH 7.0) to a final concentration of 10^9 CFU / ml and the β-galactosidase activity was observed after sonication, the BGN4-G1 showed 3.6-fold higher enzyme activity than that of the BGN4, and the BGN4-bgr showed 3.2-fold lower enzyme activity than that of the BGN4. It is considered that the bifidobacterial strains used in the LI alleviation experiment were well established according to the degree of β-galactosidase activity of the bifidobacteria. Bacterial strains used in this study are listed in Table 4.1.

A


1.0
0.8
0.6
0.4
0.2
0.0
U / ml
2.5
2.0
1.5
1.0
0.5
0.0
OD600
12
18
24
30
36
42
Incubation time (h)

B


0.8
0.6
0.4
0.2
0.0
U / ml
2.0
1.5
1.0
0.5
0.0
OD600
12
18
24
30
36
Incubation time (h)

C

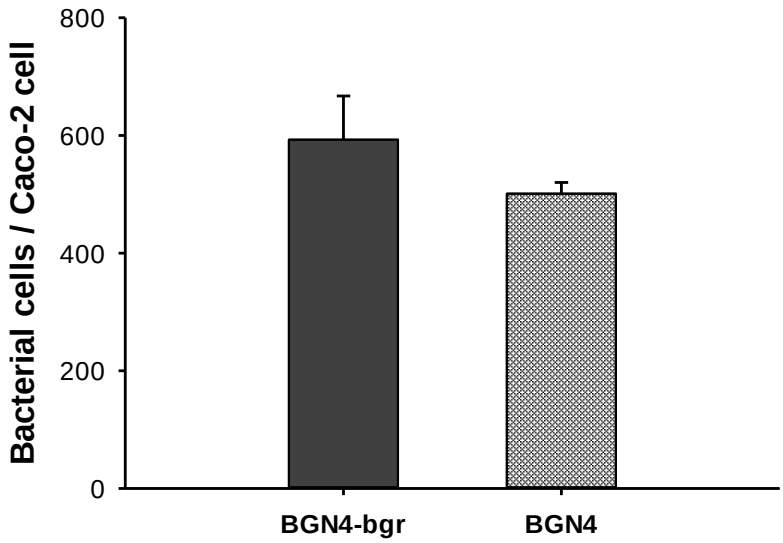


Fig. 4.1 Comparison of the β-galactosidase activity, growth rate and colon cell adhesive ability between BGN4 and BGN4-bgr

BGN4 and BGN4-bgr were cultured up to 36 (B) or 42 h (A) in MRS medium in which glucose (A) or lactose (B) was used as a carbon source, and their β-galactosidase activity and OD_{600} for the growth rate were measured at incubation times from 12 to 36 (B) or 12 to 42 h (A). In the (A) and (B), the black bar, closed circle or closed triangle represents BGN4-bgr and the white bar, open circle or open triangle represents BGN4. The β-galactosidase activity was determined at 37℃ in 50 mM phosphate buffer (pH 7.0) with ρNPG as a substrate. The colon cell adhesive ability was expressed as the number of bacteria attached to one caco-2 cell (C).

Table 4.1 Bacterial strains used in this study

Bacterial strain	Description	Source or reference
BGN4-G1	*B. bifidum* BGN4 harboring pB-G1 which is a pBES2 derivate containing β-galactosidase-expression cassette	Lab stock
BGN4	Wild-type *B. bifidum* BGN4	Isolated from breast-fed infant feces (Park et al., 1999)
BGN4-pBES2	*B. bifidum* BGN4 harboring pBES2	Lab stock
BGN4-bgr	*B. bifidum* BGN4 with reduced β-galactosidase activity by chemical mutagen.	This work

Table 4.2 Mutation of putative β-galactosidase genes in BGN4-bgr

β-galactosidase gene			
Number of Putative β-galactosidase genes	Location		Mutation
	Begin	End	DNA change
1	170425	174300	-
2	190263	192443	-
3	575594	581401	577838 G→A
4	1595246	1597315	1595952 C→T
5	1712272	1715430	-

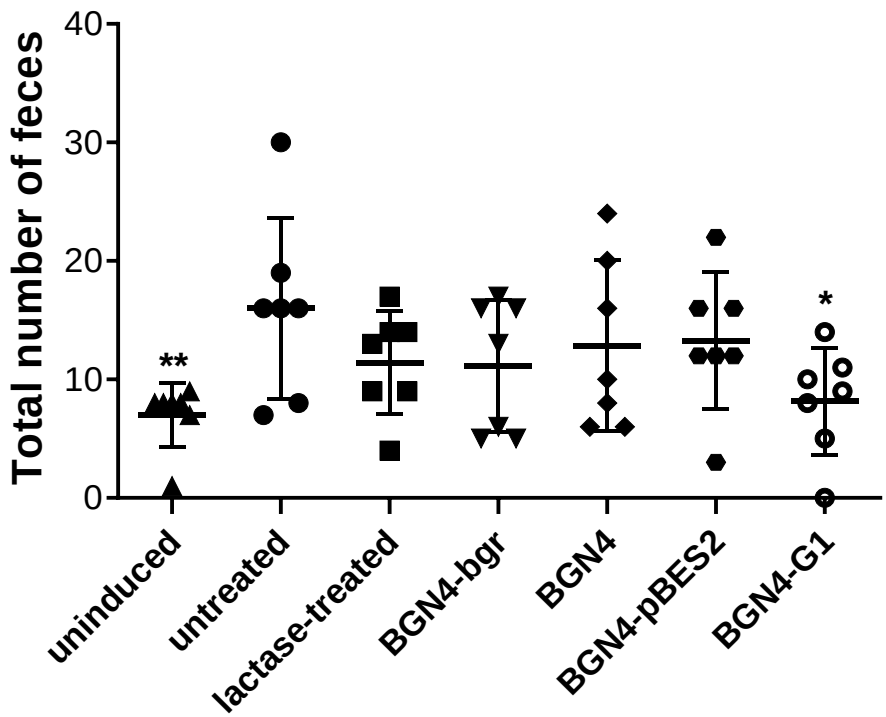
4.3.2 Stool frequency and total feces weight for 6 h after lactose administration

Seven experimental mice groups were exposed to lactose challenge after a daily intake of bacteria or normal saline (NS) for 2 weeks. The seven experimental groups were as follows: 1) a group treated with wild-type BGN4, 2) a group treated with BGN4 containing pBES2 which is the *Bifidobacterium-E. coli* shuttle vector used for G1 subcloning, 3) a group treated with BGN4-bgr (β-galactosidase reduced BGN4), 4) a group treated with BGN4-G1 (β-galactosidase over-expressing BGN4), 5) LI-induced, but untreated group, 6) a group treated with lactase just prior to LI challenge, and 7) LI-uninduced group. The stool frequencies were recorded, and the feces were collected and weighed until 6 h after the lactose challenge.

For the stool frequency, the lactase-treated group, BGN4-bgr, BGN4, and BGN4-pBES2 administration groups showed a tendency to have a decreased number of feces, but it was not a statistically significant difference compared with the untreated group (Fig. 4.2.A). On the other hand, in the case of the BGN4-G1 group, the number of stools was significantly decreased. There was no significant difference in the BGN4-G1 group compared to the uninduced group, indicating that the LI symptoms were alleviated to a level similar to the uninduced group.

In all the groups, the total feces weight tended to decrease compared to the untreated group, but only the uninduced group, lactase-treated group, and BGN4-G1 administration group were statistically significant (Fig. 4.2.B). In particular, the BGN4-G1 group had a greater difference in significance than that of the lactase-treated group and did not differ significantly from the uninduced group, indicating that the degree of LI alleviation in the BGN4-G1 group was greater than that in the lactase-treated group and even reached the LI-uninduced level.

A


Total number of feces
40
30
20
10
0
**
*
uninduced
untreated
lactase-treated
BGN4-bgr
BGN4
BGN4-pBES2
BGN4-G1

B

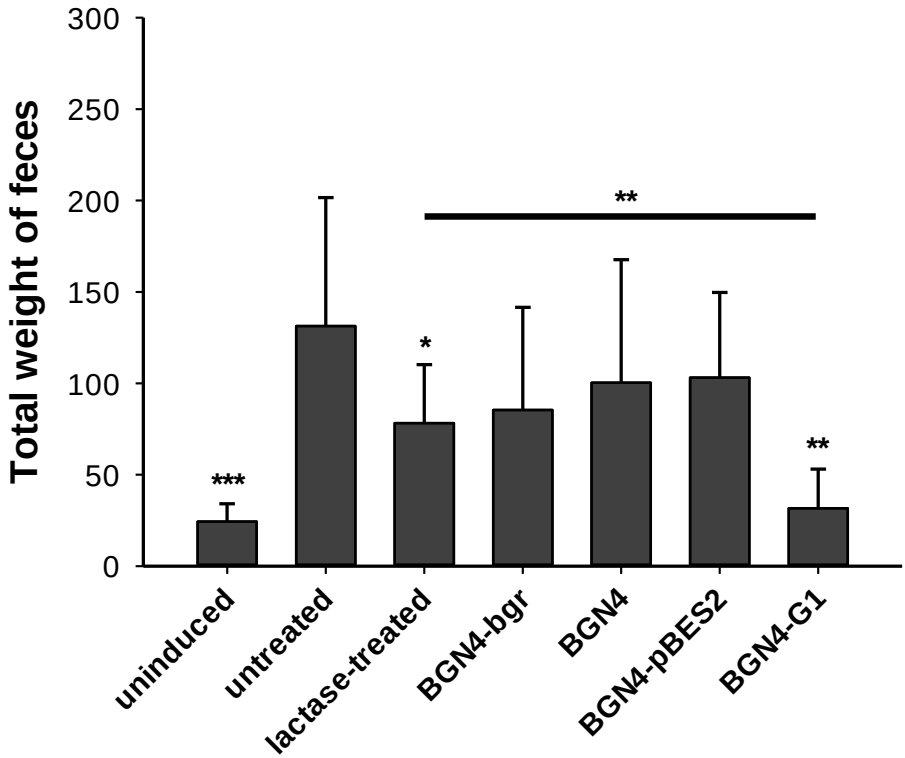


Fig. 4.2. Stool frequency and total weight of feces within 6h post-lactose challenge in mice

Total number of feces were recorded (A) and weighed (B) for each mouse individual until 6 h after lactose challenge.

4.3.3 Intestinal motility after lactose challenge

From the result shown in Fig. 4.3, a significant decrease in the running distance of the charcoal particle in both the BGN4 and BGN4−G1 groups compared with the untreated group was observed although it did not reach the level of the uninduced group. Unlike the BGN4 and BGN4−G1 groups, there was no suppression of intestinal motility in the BGN4−bgr group.

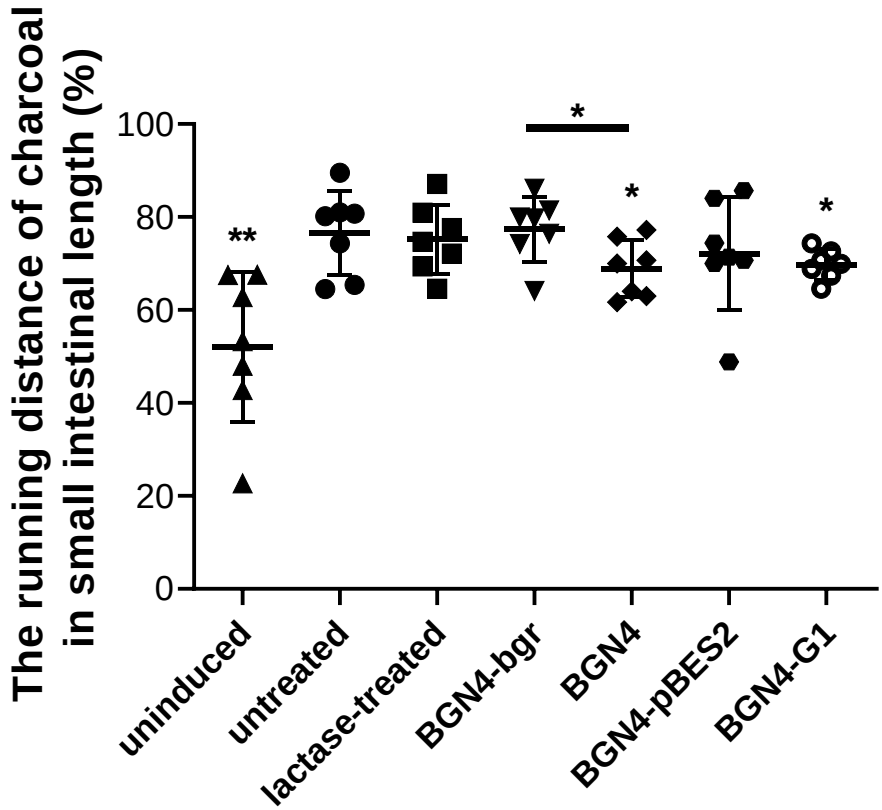


Fig. 4.3 Intestinal motility after lactose challenge in mice

Intestinal motility was expressed as percentage of the running distance of charcoal particle in total small intestinal length.

4.3.4 Effect on lactose metabolism

The metabolism of lactose in the small intestine of mice can be measured through the blood glucose after an acute lactose challenge (During et al., 1998). The difference in blood glucose between the baseline and the value after the 30 min post-lactose is shown in Fig. 4.4. As expected, the blood glucose did not rise in the uninduced group but was somewhat lower or similar to the baseline. The other mice groups had an overall elevation in the plasma glucose, suggesting that the lactose intolerance model mice used in this study do metabolize lactose in the small intestine to some extent. The change in the plasma glucose level tended to be slightly higher in the mice groups fed lactase or bifidobacteria compared to the untreated group but was not statistically significant. The lactase-treated group was thought to be significantly different from the untreated group because the mice were directly administered with a highly active lactase just before the lactose challenge, but the result was unexpected. Notably, the bifidobacteria, which mainly live in the colon rather than in the small intestine, seemed to be slightly involved in the lactose metabolism of the small intestine because the blood glucose was increased to a level similar to the lactase-treated group in all the *Bifidobacterium*-fed groups, although it was not significant.

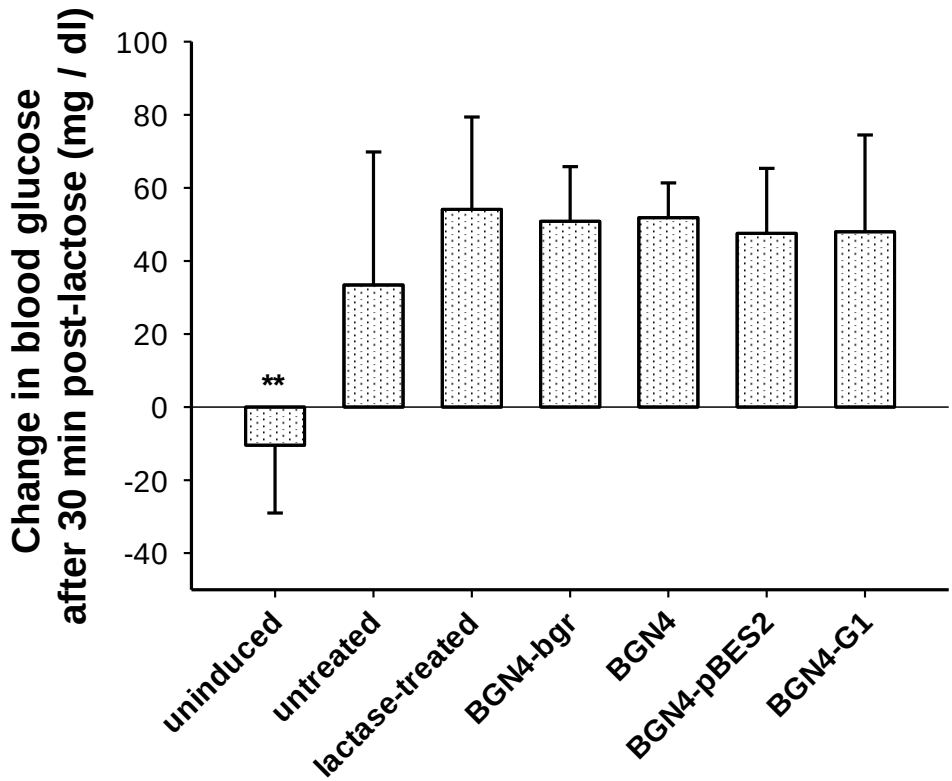


Fig. 4.4. Change in plasma glucose after the ingestion of lactose in mice fasted overnight

Fasting plasma glucose level, as the baseline, was measured in tail vein blood just before lactose administration. After the lactose challenge, the plasma glucose was again obtained from the tail-vein blood 30 min later. Change in plasma glucose was expressed as the difference in blood glucose between the baseline and the value after 30 min post-lactose.

4.3.5 Small intestinal β-galactosidase activities

In this study, LI symptoms were alleviated with the increasing β-galactosidase activity of bifidobacteria. To investigate the mechanism of the results, the β-galactosidase level in the small intestine of mice after 6 h post-lactose was measured. This value may reflect the β-galactosidase activities of the small intestine itself and the bacteria present in the small intestine such as *Lactobacillus*, *Lactococcus*, etc. As expected, the lactase fed group showed a significantly increased β-galactosidase activity compared with that of the untreated group (Fig. 4.5). The bifidobacteria administration groups except for the BGN4-pBES2 group showed a slightly higher enzyme activity than that of the untreated group, but the difference was not significant. In addition, there was no observed tendency in the result based on the β-galactosidase activity of the bifidobacteria. Taken together with the results of the plasma glucose levels (Fig. 4.4), this may suggest that the mechanism of LI alleviation as the increases of β-galactosidase activity from the bifidobacteria is not much related to the lactose metabolism in the small intestine by the β-galactosidase activities of the small intestine itself and the bacteria present there such as *Lactobacillus*, *Lactococcus*, etc.

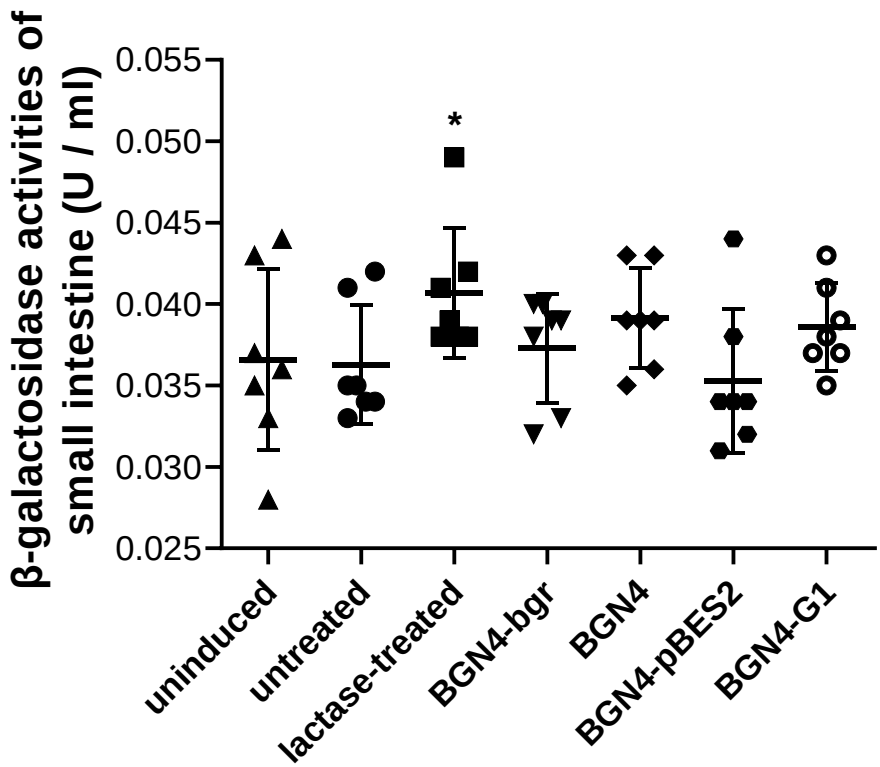


Fig. 4.5. Small intestinal β-galactosidase activity in mice

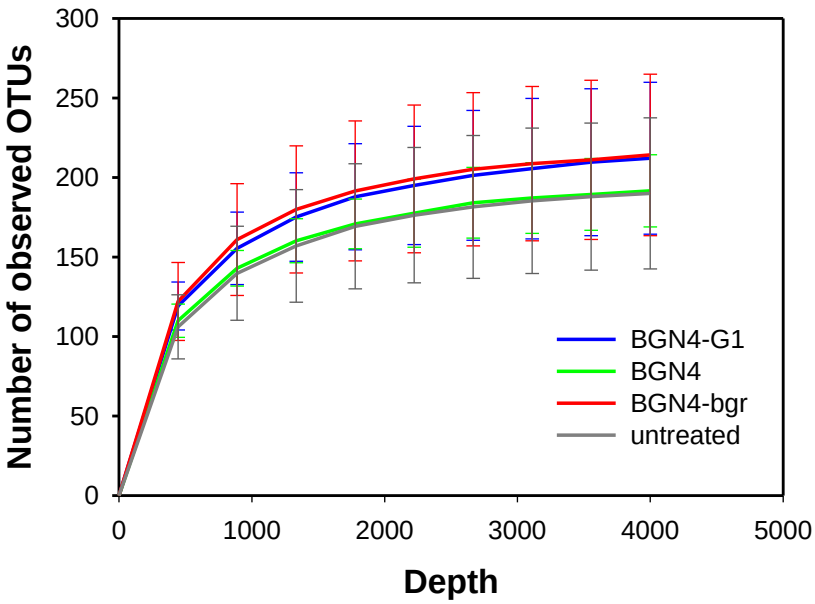
After 6 h post-challenge, 10 cm of small intestine near the stomach were anatomized out and homogenized with ten times volume of ice-cold 0.9% NS. Then, the cell and tissue debris were spun down, and the supernatant was used to measure the β-galactosidase activity. The β-galactosidase activity was determined at 37℃ in 50 mM phosphate buffer (pH 7.0) with ρNPG as a substrate.

4.3.6 Microbial diversity

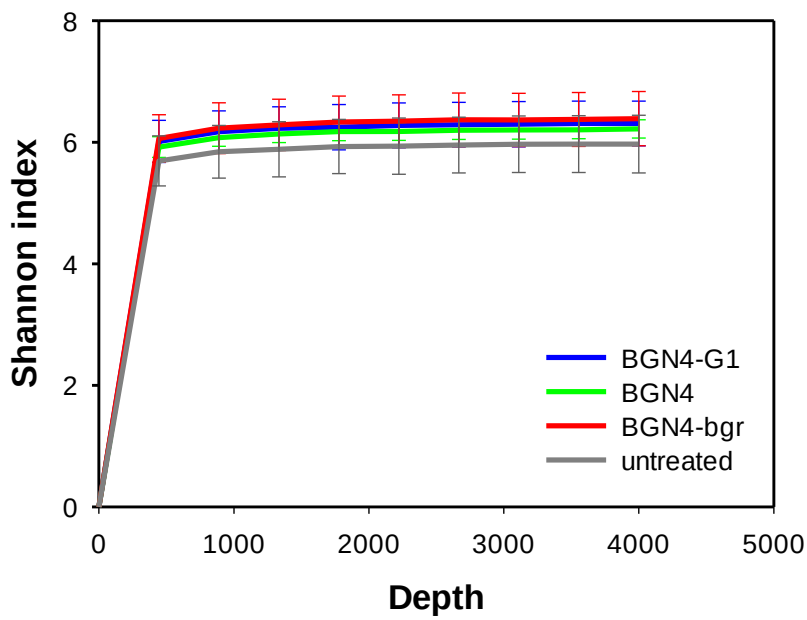
From the above results, it was found that a better lactose tolerance by the β-galactosidase-overexpressing bifidobacteria was not significantly associated with lactose metabolism in the small intestine. Therefore, it was necessary to explore the mechanisms of LI alleviation from other aspects. Previous studies have reported that LI is also associated with intestinal flora (Deng et al., 2015; Kopp-Hoolihan, 2001). Based on this viewpoint, a gut microbiome analysis from the feces of the mice fed BGN4-bgr, BGN4, BGN4-G1 or NS for two weeks was performed to investigate the LI alleviation mechanism.

First, the results for alpha-diversity (within samples) are shown in Fig. 4.6. The community richness was assessed by the number of observed OTUs (Fig. 4.6.A), Shannon index (Fig. 4.6.B) and Faith's phylogenetic diversity (Fig. 4.6.C), and the evenness was presented by Pielou's evenness index (Fig. 4.6.D). In all cases of richness, the lowest median value was seen in the untreated group. Especially when evaluated by the Shannon diversity index which considers both the species richness and evenness (Chen et al., 2016), the groups fed *Bifidobacterium* were tied together, and the untreated group showed a tendency of richness lower than that. Moreover, for evenness, the median value of the groups fed *Bifidobacterium* was higher than that of the untreated group.

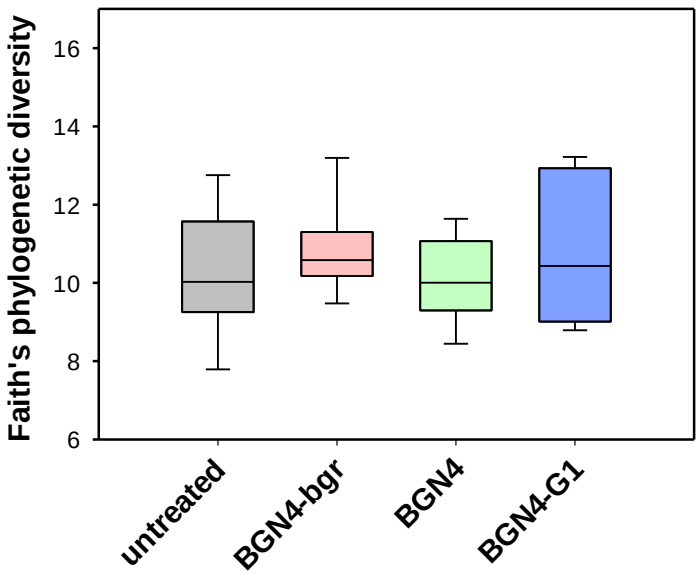
A


Number of observed OTUs
300
250
200
150
100
50
0
0
1000
2000
3000
4000
5000
Depth
BGN4-G1
BGN4
BGN4-bgr
untreated

B


Shannon index
8
6
4
2
0
0
1000
2000
3000
4000
5000
Depth
BGN4-G1
BGN4
BGN4-bgr
untreated

C


Faith's phylogenetic diversity
16
14
12
10
8
6
untreated
BGN4-bgr
BGN4
BGN4-G1

D

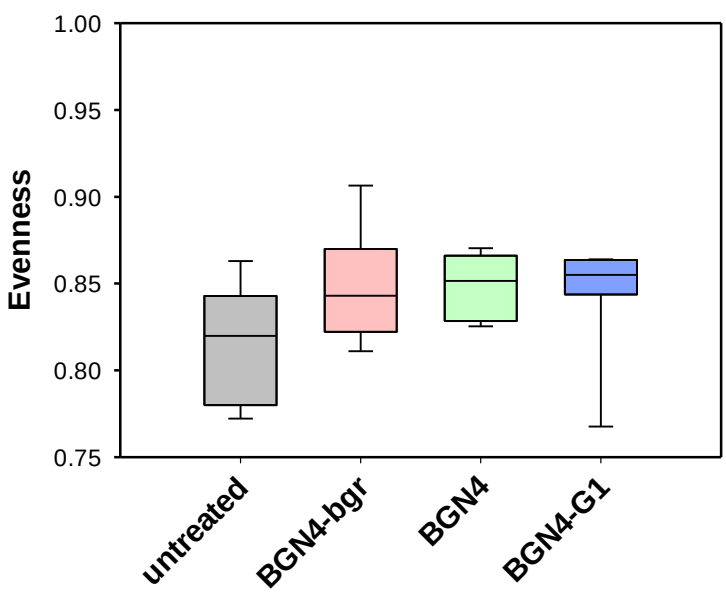

Evenness
1.00
0.95
0.90
0.85
0.80
0.75
untreated
BGN4-bgr
BGN4
BGN4-G1

Fig. 4.6 Alpha diversity in mice feces

Bacterial richness index graphs (Number of observed OTUs (A), Shannon index (B), and Faith's phylogenetic diversity (C)) and evenness index graph (Pielou's evenness index (D)) were obtained from fecal microbiome samples.

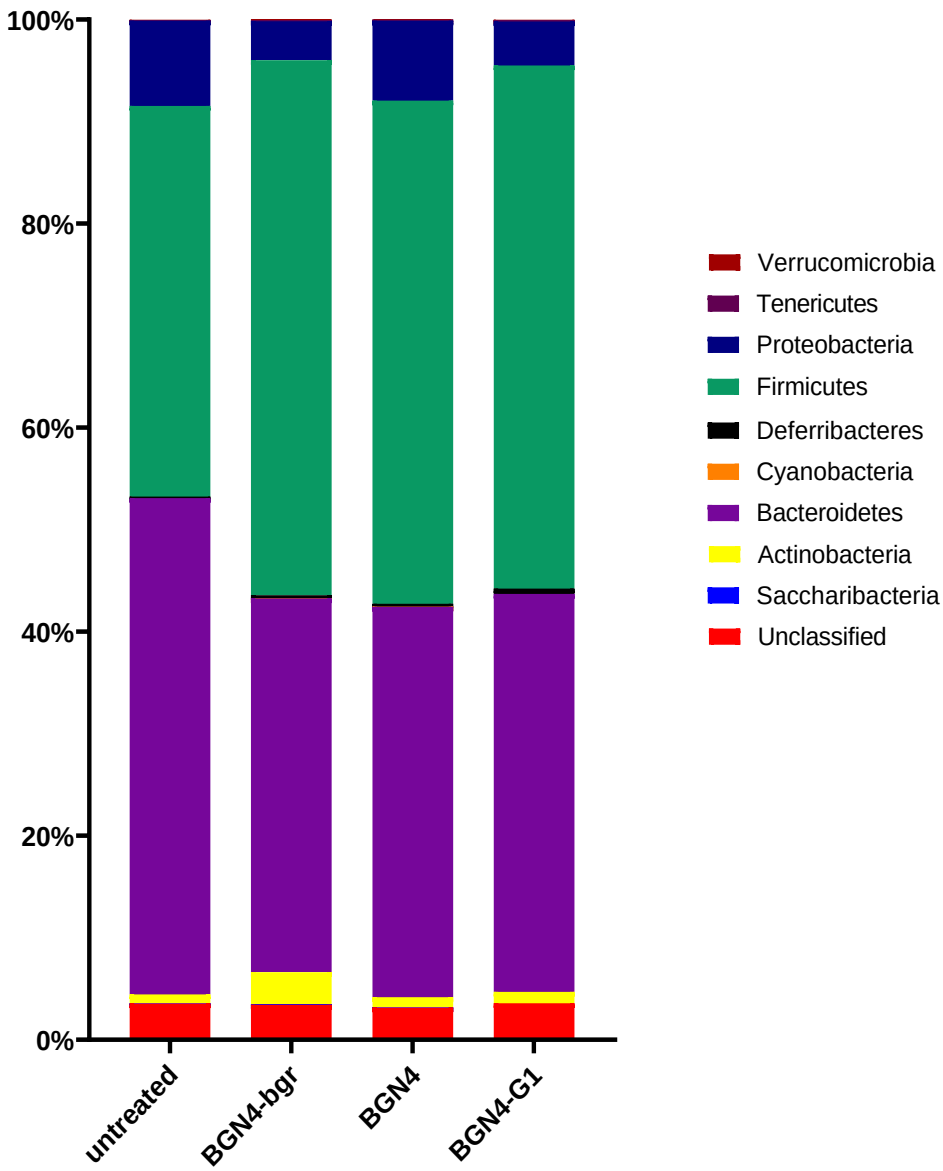
4.3.7 Bacterial OTU abundances at the phylum taxonomic level

Next, the OTU abundances for each mouse group at the phylum level were calculated and presented in Fig. 4.7. The untreated group had a lower proportion of Firmicutes and a higher proportion of Bacteroidetes than the groups fed *Bifidobacterium* although not statistically significant due to the large differences among the mouse individuals (Fig. 4.7.A). In accordance with these results, the ratio of Firmicutes / Bacteroidetes tended to be higher in the groups fed *Bifidobacterium* compared with the untreated group (Fig. 4.7.B).

The phyla Bacteroidetes and Firmicutes are generally dominant in the intestine of healthy humans (Johnson et al., 2017). It has been reported previously that the abundance of Firmicutes was less and that of Bacteroidetes was greater in IBD patients compared to healthy subjects (Marteau, 2009; Wright et al., 2015). However, the opposite result was also reported by Rooks MG *et al.* (Rooks et al., 2014). These contradictory results may simply be caused by the mouse model used. Regardless of the outcomes, the crucial point is that Firmicutes and Bacteroidetes contain both colitis- and health-associated genera (Jones-Hall and Nakatsu, 2016). Meanwhile, a previous study showed that sensitivity to lactose occurs in a high proportion of IBD (Inflammatory bowel disease) patients (Eadala et al., 2011). It is notable that the proportion of the two dominant

phylum (Firmicutes and Bacteroidetes) related to colonic health was changed from one another according to whether *Bifidobacterium* or NS was ingested, and the *Bifidobacterium*-intake groups, especially BGN4-G1 group, alleviated LI symptoms.

A


100%
80%
60%
40%
20%
0%
untreated
BGN4-bgr
BGN4
BGN4-G1
Verrucomicrobia
Tenericutes
Proteobacteria
Firmicutes
Deferribacteres
Cyanobacteria
Bacteroidetes
Actinobacteria
Saccharibacteria
Unclassified

B

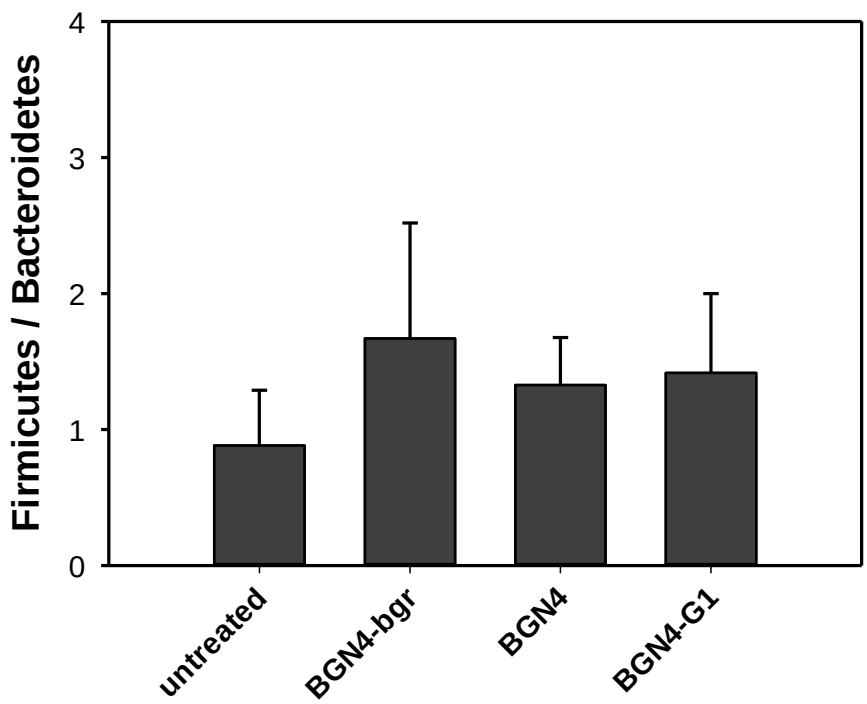


Fig. 4.7 The OTU abundances at the phylum level in mice feces

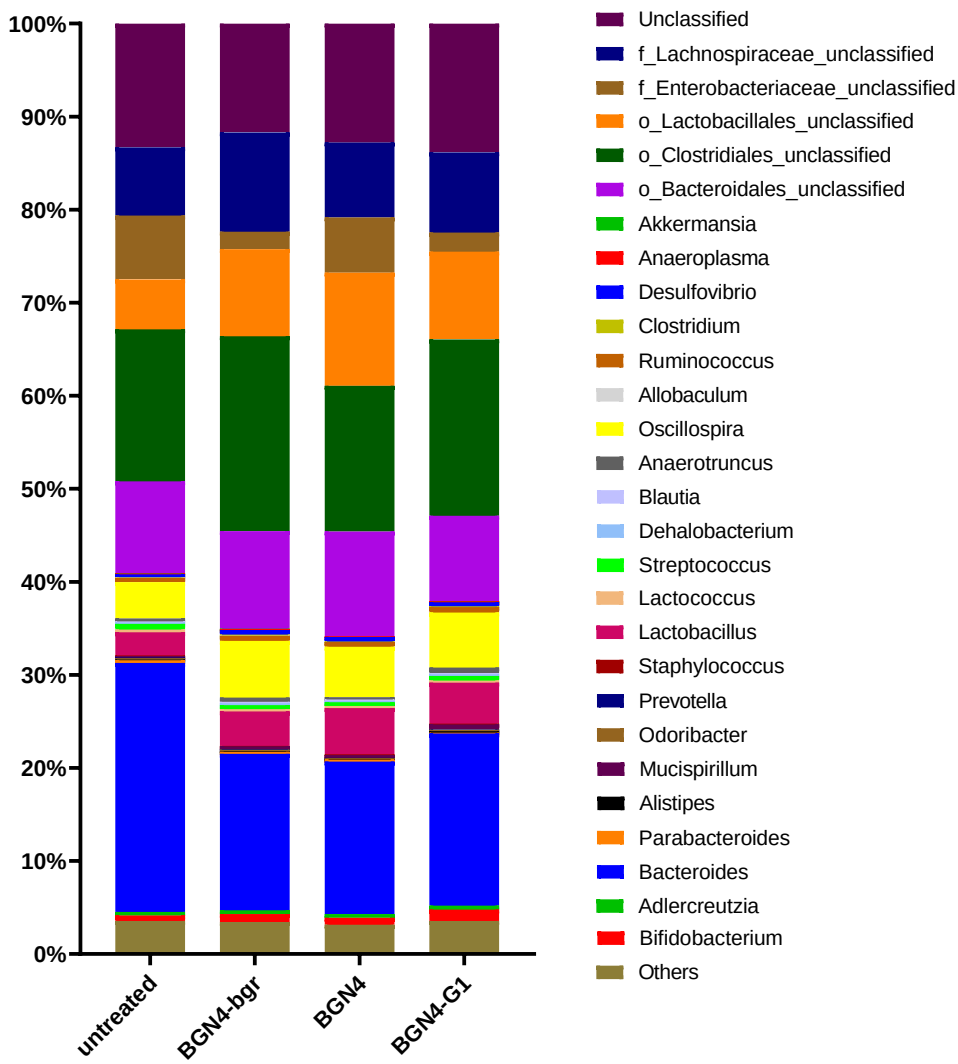
Overall microbiota composition of the average relative abundance of each group at the phyla level (A) and ratio of Firmicutes : Bacteroidetes among the groups (B) are presented.

4.3.8 Bacterial OTU abundances at the genus taxonomic level

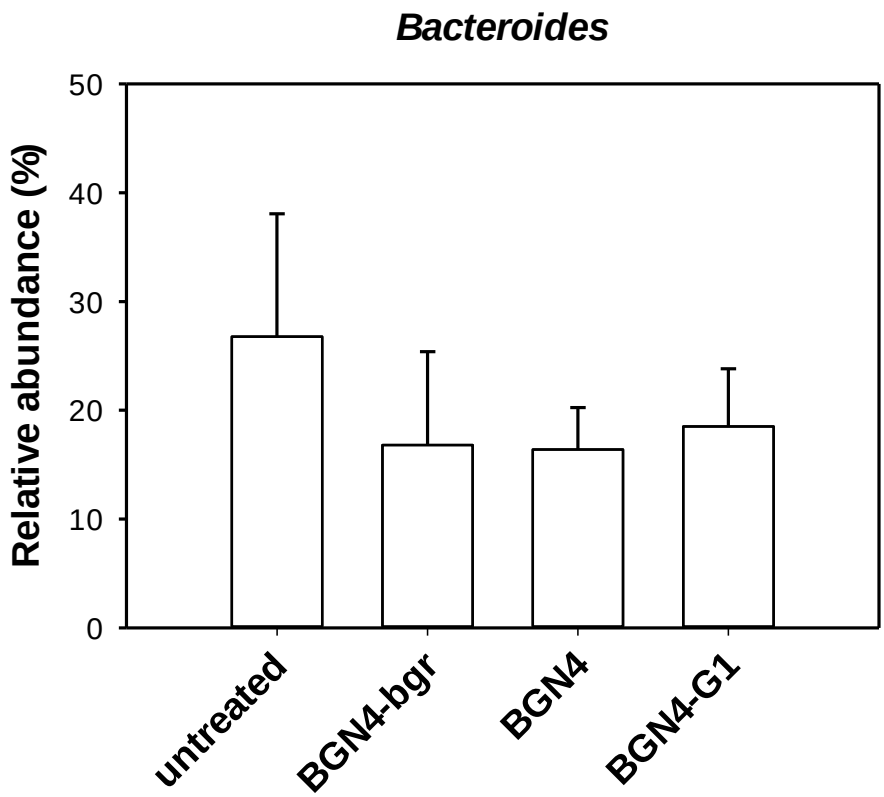
Bacterial OTU abundances for each mouse group at the genus taxonomic level are shown in Fig. 4.8. There was no significant difference, but *Bifidobacterium* and *Lactobacillus* were increased in the groups fed *Bifidobacterium* compared to the untreated group (Fig. 4.8.C, 4.8.D). It is natural that the relative abundances of *Bifidobacterium* were increased in the *Bifidobacterium*-intake groups, but the increase in *Lactobacillus* was not expected as much. This phenomenon might be due to the correlation between *Bifidobacterium* and *Lactobacillus* (Li et al., 2012). In the case of *Bifidobacterium*, although the mean value of relative abundance in the BGN4-G1 group was higher than that of the BGN4 and BGN4-bgr groups, there was no significant tendency for the colonization of *Bifidobacterium* according to the β-galactosidase activities between the *Bifidobacterium*-intake groups. The proportion of *Oscillospira* was higher in the groups fed *Bifidobacterium*, and especially in BGN4-G1, *Oscillospira* was significantly higher compared to the untreated group (Fig. 4.8.E). Recently, a meta-analysis of five microbiota studies in IBD patients reported *Oscillospira* to be significantly reduced in patients with Crohn's disease (Walters et al., 2014), which means that *Oscillospira* is positively related to gut health (Konikoff and Gophna, 2016).

Meanwhile, the abundance of *Bacteroides* was not significant but was higher in the untreated group than in the groups fed *Bifidobacterium*, which seemed to influence the proportion of Bacteroidetes at the phylum level (Fig. 4.8.B). Finally, the ratio of *Bifidobacterium* to *Enterobacteriaceae* (B/E ratio) was investigated (Fig. 4.8.F). The B/E ratio is a typical indicator for the resistance of the gastrointestinal tract against the colonization of potentially pathogenic microbes (Si et al., 2004; Wu et al., 2000). The untreated group had a lower B/E ratio than the groups fed *Bifidobacterium*, albeit not statistically significant.

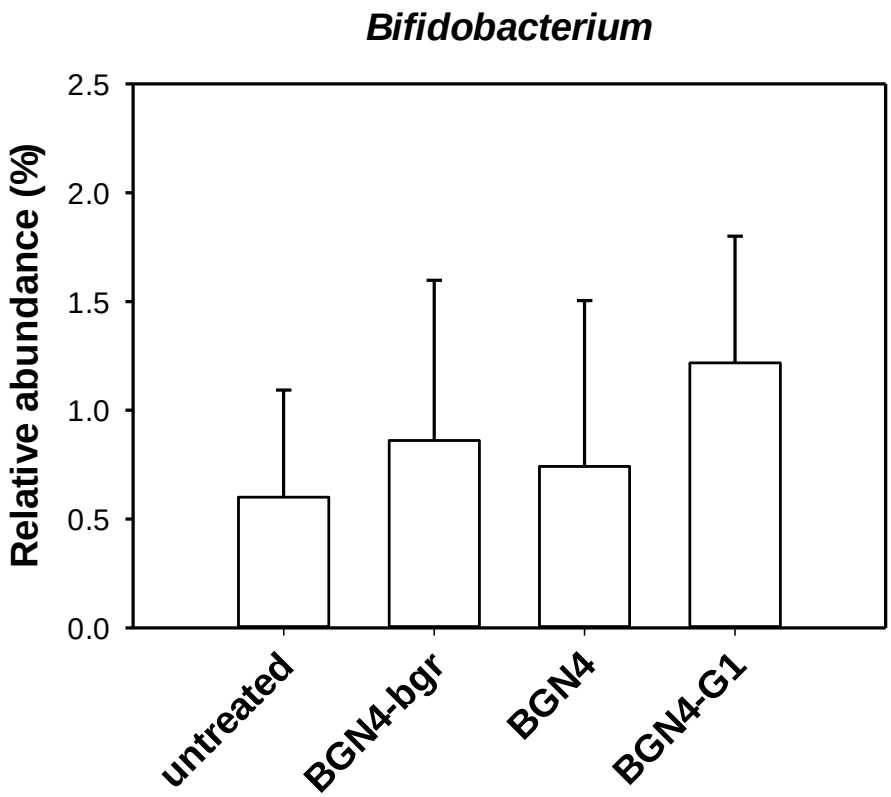
A


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
untreated
BGN4-bgr
BGN4
BGN4-G1
Unclassified
f_Lachnospiraceae_unclassified
f_Enterobacteriaceae_unclassified
o_Lactobacillales_unclassified
o_Clostridiales_unclassified
o_Bacteroidales_unclassified
Akkermansia
Anaeroplasma
Desulfovibrio
Clostridium
Ruminococcus
Allobaculum
Oscillospira
Anaerotruncus
Blautia
Dehalobacterium
Streptococcus
Lactococcus
Lactobacillus
Staphylococcus
Prevotella
Odoribacter
Mucispirillum
Alistipes
Parabacteroides
Bacteroides
Adlercreutzia
Bifidobacterium
Others

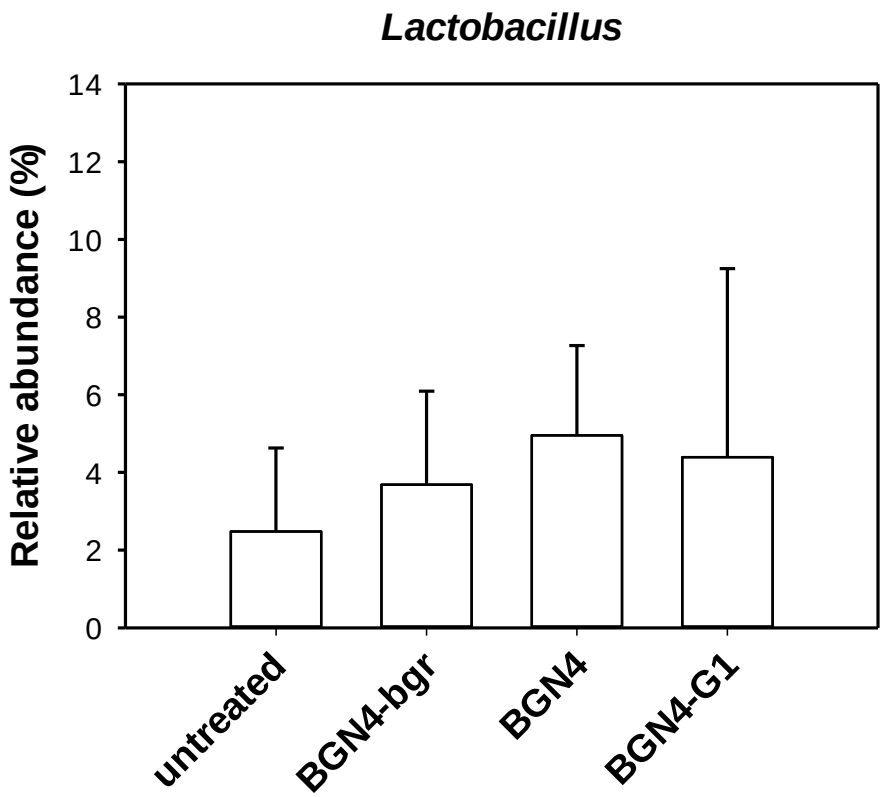
B


Bacteroides
Relative abundance (%)
50
40
30
20
10
0
untreated
BGN4-bgr
BGN4
BGN4-G1

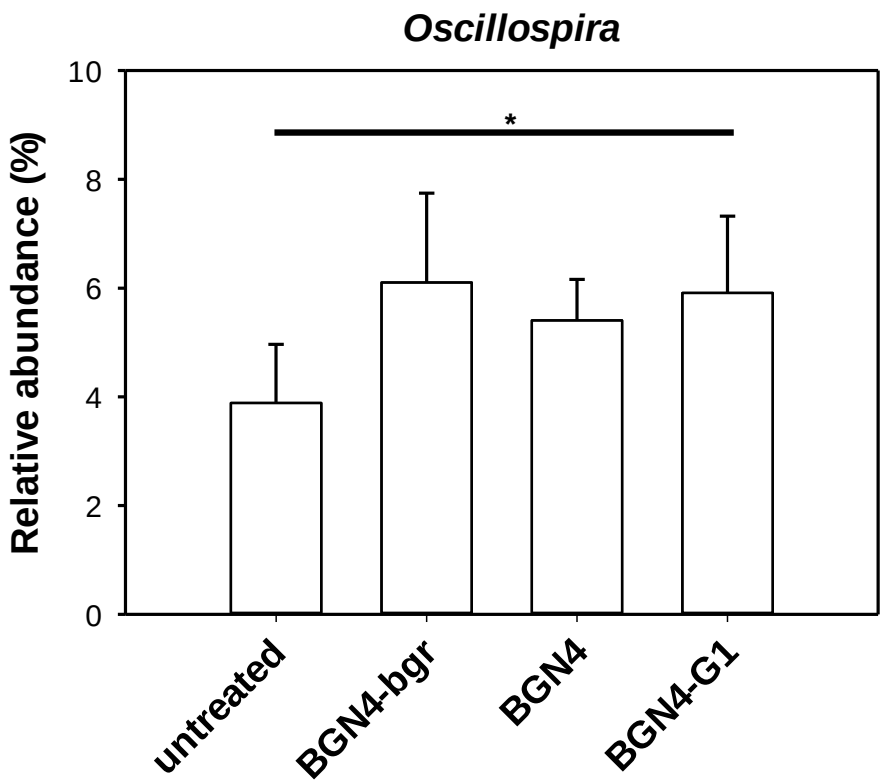
C


Bifidobacterium
Relative abundance (%)
2.5
2.0
1.5
1.0
0.5
0.0
untreated
BGN4-bgr
BGN4
BGN4-G1

D


Lactobacillus
Relative abundance (%)
14
12
10
8
6
4
2
0
untreated
BGN4-bgr
BGN4
BGN4-G1

E


Oscillospira
Relative abundance (%)
10
8
6
4
2
0
*
untreated
BGN4-bgr
BGN4
BGN4-G1

F

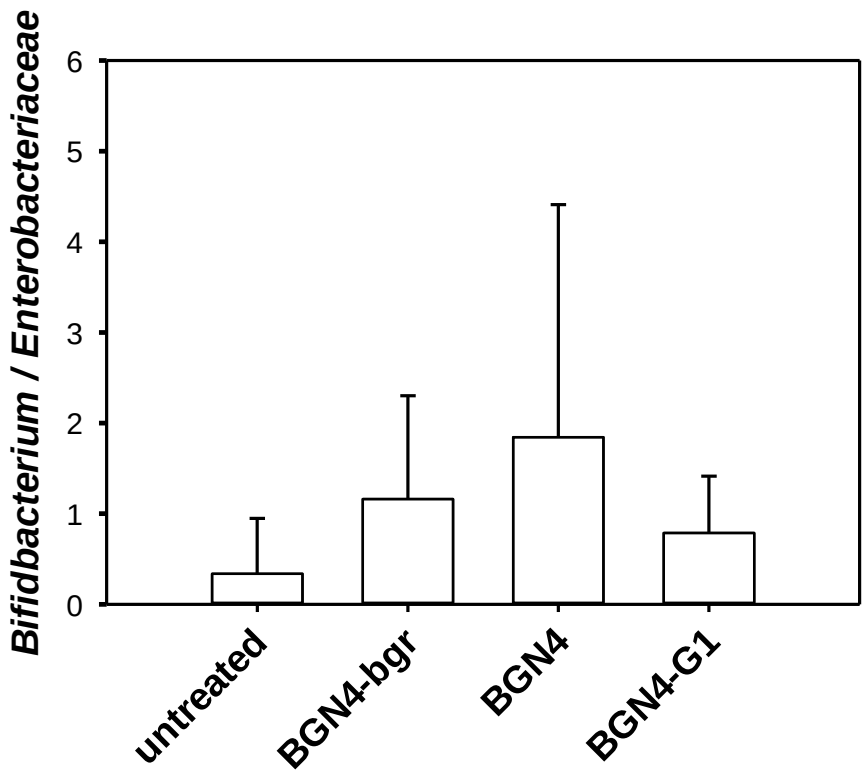


Fig. 4.8 The OTU abundances at the genus level in mice feces

Overall microbiota composition of the average relative abundance of each group at the genus level (A) and comparison of relative abundance of several genus among groups (B–F) are presented.

4.3.9 SCFAs and lactate concentration in the cecum

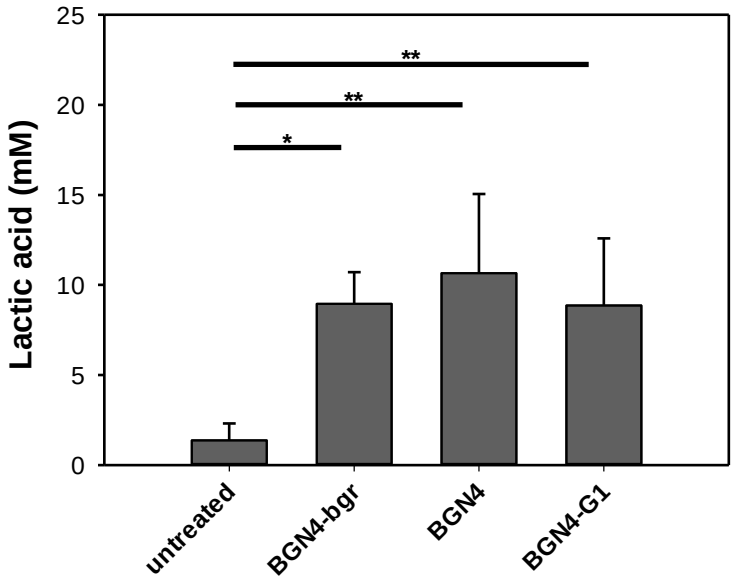
SCFAs (Short chain fatty acids) and lactate are fermentation products of the gut microbiota. Food components that are unabsorbed/undigested in the small intestine are fermented in the colon to produce SCFAs including acetate, propionate and butyrate (Russell et al., 2013). Lactate is also produced by several microbiota components such as *Bifidobacterium*, *Lactobacillus* and Proteobacteria (Flint et al., 2015). SCFAs are associated with a reduced risk of several intestinal diseases including irritable bowel syndrome and IBD (Hijova and Chmelarova, 2007).

In this study, the SCFA contents and lactate were incidentally measured in the cecum of mice fed *BGN4-bgr, BGN4, BGN4-G1* or NS for two weeks to analyze the changes in the intestinal metabolites by the administered bacteria. Because rats are cecal fermenters, unlike humans which are colonic fermenters, many mice and rat studies analyze the cecal samples to determine the amount of SCFAs (Apajalahti et al., 2002; Campbell et al., 1997; Kleessen et al., 1997). As a result, acetate and butyrate showed a tendency to increase in the BGN4-G1 and BGN4 groups compared to the untreated group although not significant, but the mean value of the BGN4-bgr group was similar to that of the untreated group (Fig. 4.9.C, 4.9.D). The lactate content of all the *Bifidobacterium*-fed groups was significantly greater than that of the untreated group, and especially,

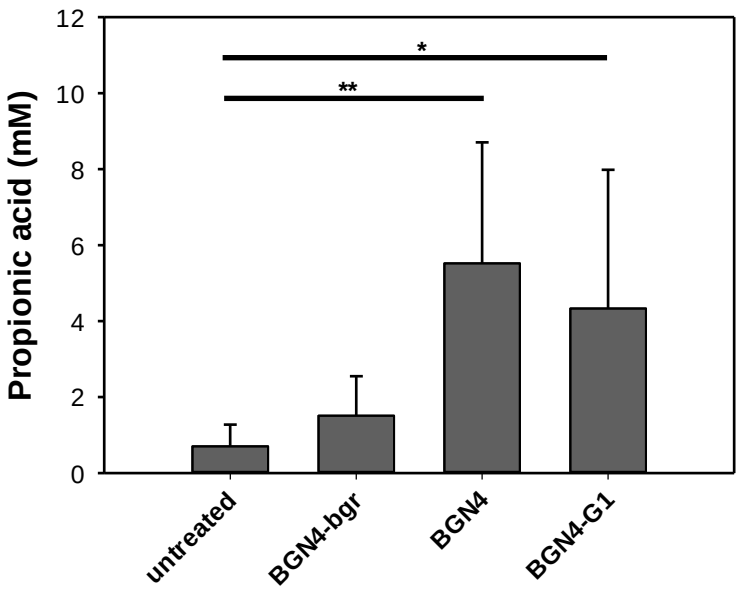
the BGN4-G1 and BGN4 groups had a *P* value of less than 0.01 (Fig. 4.9.A). In the case of propionate, the propionate concentration in the cecum of the BGN4-G1 and BGN4 groups was significantly higher than that of the untreated group, but the BGN4-bgr group did not show any significant difference (Fig. 4.9.B). Because *Bifidobacterium* and *Lactobacillus* are representative producers of lactate and acetate (Beards et al., 2010), the increase in lactate and acetate in the *Bifidobacterium*-fed groups may be due to the OTU abundance increase of *Bifidobacterium* and *Lactobacillus* in these groups, although it was not significant (Fig. 4.8). In the intestine, the produced lactate and acetate are converted to propionate and butyrate through cross-feeding by other dominant members of the gut microbiota (Beards et al., 2010). The reason for the increase of butyrate and propionate in the *Bifidobacterium*-fed groups is probably because the increased lactate and acetate in these groups were actively metabolized by other microorganisms and converted to butyrate and propionate. There is a report that SCFAs can contribute to the osmotic pressure in the colon (Vonk et al., 2012). In this study, although total SCFAs in the *Bifidobacterium*-intake groups were increased compared to the untreated group, LI symptoms were rather alleviated in the *Bifidobacterium*-intake groups. This result may indicate that the balanced ratio of the SCFAs is more important than the total amount of SCFAs or there are other alleviation factors,

which can offset the increased osmotic load caused by SCFAs, in the *Bifidobacterium*–intake groups.

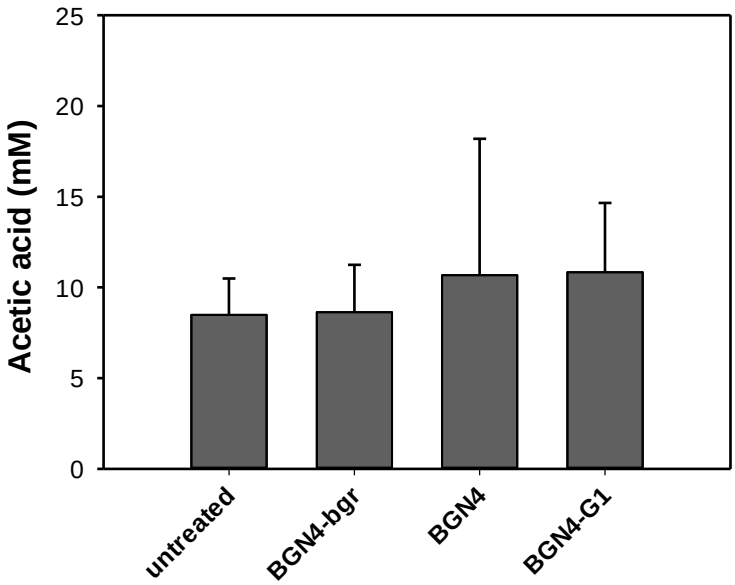
A


Lactic acid (mM)
25
20
15
10
5
0
**
**
*
untreated
BGN4-bgr
BGN4
BGN4-G1

B


Propionic acid (mM)
12
10
8
6
4
2
0
*
**
untreated
BGN4-bgr
BGN4
BGN4-G1

C


Acetic acid (mM)
25
20
15
10
5
0
untreated
BGN4-bgr
BGN4
BGN4-G1

D

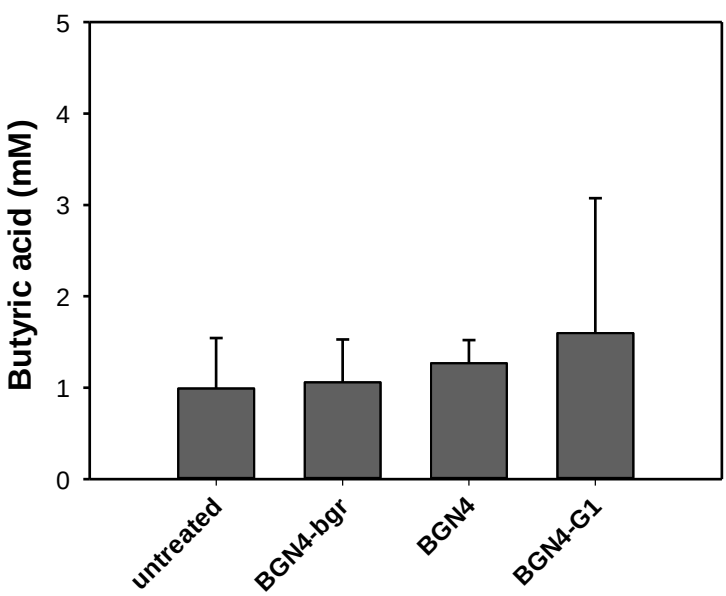

Butyric acid (mM)
5
4
3
2
1
0
untreated
BGN4-bgr
BGN4
BGN4-G1

Fig. 4.9 Concentration of lactate and SCFAs in 100 mg of mice caecum

The cecal samples (100 mg) were homogenized, acidified with 25% metaphosphoric acid and centrifuged. The supernatant was analyzed by HPLC to determine the SCFA and lactate concentrations.

4.4 Discussion

Probiotics administration has received attention as one of the LI treatments. However, because of the contradictory results of previous studies, no clear conclusion has been drawn as to whether probiotics have an effect on LI. I thought that the conflicting results may be due to the different β-galactosidase activities of the probiotics strains used in each study and devised experiments using bifidobacteria, which have a relatively high β-galactosidase activity among the probiotics, with different β-galactosidase levels to confirm the hypothesis. Therefore, the present work evaluated *in vivo* the effect of the β-galactosidase activity of bifidobacteria on LI alleviation by administering β-galactosidase reduced bifidobacteria, wild-type bifidobacteria and β-galactosidase over-expressing bifidobacteria.

B. bifidum BGN4 was selected as a wild-type *Bifidobacterium* strain used in this study because of its genetical accessibility and outstanding colon cell binding ability among other bifidobacteria. Previously, I developed a recombinant BGN4 strain expressing exogenous β-galactosidase (BGN4-G1), which shows an average of 3.5 times higher enzyme activity than that of the wild-type and decided to use it as a β-galactosidase over-expressing BGN4 in

the present work. In the case of a β-galactosidase reduced BGN4, a strain (BGN4-bgr) showing approximately 3.2 times lower enzyme activity than that of the wild-type was developed by randomized mutagenesis. Through these processes, the β-galactosidase reduced, wild-type, and β-galactosidase over-expressing bifidobacteria were prepared for use in this study.

Balb/c mice known as the LI model mice have been shown to have a high lactase activity in the small intestine before weaning and then to decrease significantly to a very low level after weaning (9 weeks old), as in adult-type hypolactasia in humans (He et al., 2005; Li et al., 2012). In this study, the Balb/c mice were grouped and orally administered with different live bacterial suspensions or NS for 2 weeks. After that, the mice were challenged with lactose, and symptoms of lactose intolerance were observed. As a result, the BGN4-G1 administration group showed a remarkable decrease in stool frequency (Fig. 4.2.A) and total feces weight (Fig. 4.2.B) for 6 h post-challenge and suppression of intestinal motility (Fig. 4.3) compared with the untreated group. The LI alleviation effect in the BGN4-G1 administration group was greater than that in the lactase-treated group and even reached a LI-uninduced level. Exogenous β-galactosidase has traditionally attracted attention as a strategy to manage LI subjects and are currently on the market. To compare probiotics with exogenous β-galactosidase for LI alleviation, a commercially available lactase tablet was used as a positive control

in this study. As mentioned above, the BGN4-G1 intake group showed significantly better LI-alleviating effects than that of the lactase-treated group, which is meaningful. The orally intake of β-galactosidase is inconvenient because it must be taken every time whenever consuming lactose-containing foods and is not very effective due to rapid degradation of the enzyme activity (Zhang et al., 2011). In this study, lactose challenge was done 12 h after the last administration of *Bifidobacterium*; however, it was found that the LI alleviating effects of the *Bifidobacterium* highly expressing β-galactosidase were better than that of the lactase treatment despite being administered just before the challenge. Meanwhile, the stool frequency and total feces weight of the BGN4 and BGN4-bgr administration groups tended to decrease compared to the untreated group, but no significant difference was found. In the case of intestinal motility, in contrast to the BGN4-bgr group, the BGN4 administration group was significantly inhibited in intestinal motility compared to the untreated group. This suggests that the LI alleviation effect is improved as the β-galactosidase activity of the bifidobacterial is increased.

To explore the mechanisms for the demonstrated better lactose tolerance of the bifidobacteria highly expressing β-galactosidase, the lactose metabolism level in the small intestine was first monitored. Because elevated levels of plasma glucose after 30 min post-lactose (Fig. 4.4) and β-galactosidase activities of the small intestine (Fig.

4.5) in the bifidobacteria-fed groups were not significantly different compared to the untreated group, the mechanisms of LI alleviation by the β-galactosidase level of bifidobacteria do not seem to have much to do with the lactose metabolism in the small intestine by β-galactosidase activities of the small intestine itself and the bacteria present there.

In previous studies, it has been reported that LI is also associated with gut microflora (Deng et al., 2015; Kopp-Hoolihan, 2001). Based on this viewpoint, gut microbiome analysis from the feces of mice fed BGN4-bgr, BGN4, BGN4-G1 or NS for two weeks was performed to investigate the LI alleviation mechanism. As a result, the *Bifidobacterium*-fed groups showed better species richness and evenness than that of the untreated group (Fig. 4.6). In the OTU abundances at the phylum level, the *Bifidobacterium*-fed groups had a higher proportion of Firmicutes and a lower proportion of Bacteroidetes than that of the untreated group although not statistically significant (Fig. 4.7). In the OTU abundances at the genus level, *Bifidobacterium, Lactobacillus*, *Oscillospira* and the B/E ratio tended to occupy a higher proportion in the *Bifidobacterium-fed* groups compared to the untreated group, and especially in the BGN4-G1 group, *Oscillospira* was significantly higher than in the untreated group. Meanwhile, *Bacteroides* was not significant but was lower in the groups fed *Bifidobacterium* compared to the untreated group (Fig. 4.8). The important point of all these gut microbiome

results is that the changed factors have all been reported to be associated with intestinal health (Jones-Hall and Nakatsu, 2016; Konikoff and Gophna, 2016; Si et al., 2004; Walters et al., 2014; Wu et al., 2000), and the changes in these factors were clearly differentiated according to whether mice were fed *Bifidobacterium* or NS.

Because SCFAs and lactate are fermentation products of gut microbes, the contents of SCFAs and lactate were incidentally measured in the cecum of mice fed BGN4-bgr, BGN4, BGN4-G1 or NS for two weeks. In all cases, BGN4-G1 and BGN4 had significantly higher or a higher tendency compared to the untreated, but BGN4-bgr did not show a significant difference despite the relative increase, or the mean value was similar to the untreated group (Fig. 4.9). Although the tendency of the gut microbiome proportion between the BGN4 strains with different β-galactosidase levels was not revealed conspicuously and significantly, the SCFA and lactate contents of the BGN4-G1 and BGN4 administration group, which had effects on LI alleviation, tended to be different from the BGN4-bgr group.

The gut microbiome analysis did not clearly reveal the mechanism of the better lactose tolerance by the β-galactosidase level in the bifidobacteria. However, some meaningful results were derived as follows: 1) The proportion of several microorganisms associated with intestinal health tended to be differentiated between the

Bifidobacterium-fed groups and the untreated group, 2) The bifidobacterial proportion in the BGN4-G1 group tended to be increased compared to the other *Bifidobacterium*-fed groups, 3) The balance of SCFAs and lactate was differentiated between the *Bifidobacterium*-fed groups and the untreated group, 4) The rates for the change in SCFAs and lactate were grouped in a similar pattern in the BGN4-G1 and BGN4 groups except for the BGN4-bgr group.

Chapter 5.

Evaluation of SOD and catalase as a food-grade selection marker for *Bifidobacterium* strain

5.1 Introduction

Currently, the application of genetically modified (GM) microorganisms to food and health is strictly regulated (Pedersen et al., 2005). In order for GM microorganisms to be used in the food and health industries, host strains used for genetic engineering must have the GRAS (Generally recognized as safe) status, and genetic engineering should be done with a food-grade cloning system. A food-grade vector means that it contains only the DNA from homologous hosts or GRAS organisms and is not dependent on antibiotic resistance markers (Landete, 2017).

Because lactic acid bacteria (LAB) including the *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus,* *Pediococcus*, and *Oenococcus* species are generally considered as safe (Stiles and Holzapfel, 1997), there have been many studies to develop a food-grade vector for LAB. Especially, *Lactobacillus* and *Lactococcus* species have been mainly used as a host for genetic engineering with a food-grade cloning system (Jeong et al., 2006; Li et al., 2011; Liu et al., 2005; Nguyen et al., 2011). Lately, there have been various applications of the food-grade cloning system such as expressing enzymes required for cheese production in LAB and using LAB as mucosal delivery vectors for therapeutic proteins and DNA vaccines

(Alvarez-Sieiro et al., 2014; Bermúdez-Humarán et al., 2011; Joutsjoki et al., 2002; Steidler et al., 2003; Wegmann et al., 1999).

Because *Bifidobacterium* is also a representative of probiotics and GRAS microorganisms, it is an another appealing host for a food-grade vector (Cano-Garrido et al., 2015). However, there are some limitations to actively perform genetic engineering studies with bifidobacteria. It should be noted that bifidobacteria have a strict anaerobic metabolism and a multilayered and complex cell wall (FISCHER et al., 1987) and restriction-modification systems making it difficult to use them in experiments and to achieve a high transformation efficiency (Park et al., 2018). Because of these limitations, food-grade vectors for bifidobacteria have not been developed yet to my knowledge. Nevertheless, because bifidobacteria mainly reside in the colon (De Vuyst and Leroy, 2011; Gibson and Roberfroid, 1995; Reuter, 2001), when applied as an oral vector, they will have their own value compared with *Lactobacillus* and *Lactococcus*, which are relatively more common in the small intestine (Reuter, 2001).

Bifidobacteria are lactic acid-producing, gram-positive and obligate anaerobic bacteria (Wei et al., 2007). Because superoxide dismutase (SOD) and catalase are important components of the antioxidant defense system in cells against oxygen, these enzyme activities are deficient or absent in obligate anaerobic bacteria including bifidobacteria (Shimamura et al., 1992). Interestingly, several

studies have shown that the expression of SOD and catalase in bifidobacteria dramatically increases the survival rate up to $10^4 - 10^5$ fold under oxidative stress (He et al., 2012; Zuo et al., 2014). Thus, the aim of this study was to evaluate the feasibility of using the SOD and catalase genes as a selection marker for the construction of a food-grade vector for bifidobacteria which has not been developed so far.

5.2 Materials and Methods

5.2.1 Bacterial strains, plasmid DNA and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 5.1. *E. coli* were grown at 37℃ in Luria-Bertani (LB) medium (BD Difco™, Sparks, MD, USA) with shaking at 150 rpm. Bifidobacteria were cultured at 37℃ in Man Rogosa Sharpe (MRS) medium (BD Difco™) containing 0.05% L-cysteine·HCl and 10 or 30 μM hematin (final concentration) for some of the experiments.

Antibiotics were used at the following concentrations: 100 μg/ml ampicillin (BIO BASIC INC., Markham, ON, Canada) for *E. coli* and 3 μg/ml chloramphenicol (Duchefa, Haarlem, The Netherlands) for bifidobacteria.

Table 5.1 Bacterial strains and plasmids used in this study

Bacterial strain	Description	Source or reference
Escherichia coli DH5 α		Lab stock
Bifidobacterium bifidum BGN4		Isolated from breast-fed infant feces (Park et al., 1999b)
BGN4-SK		This work
Plasmid		
pBES2	7.6 kbp, Ap^R, Cm^R *Bifidobacterium* – *E. coli* shuttle vector	(Park et al., 2003)
pB-Kat	9.2 kbp, Ap^R, Cm^R, pBES2 derivate, containing Pgap-Kat expression cassette	This work
pB-SK	10.1 kbp, Ap^R, Cm^R, pBES2 derivate, containing Pgap-Kat-Phup-SOD-Thup expression cassette	This work

5.2.2 DNA molecular cloning and transformation

The target DNA fragments were amplified using TAKARA PrimeSTAR GXL DNA Polymerase (TAKARA, Kusatsu, Japan), and each primer set is listed in Table 5.2. First, the DNA fragment encoding the Pgap-Kat expression cassette was PCR-amplified from the Pgap-Kat-Phup-SOD-Thup expression cassette (Zuo et al., 2014) and cloned into pBES2 via *AscI* and *FseI* to construct pB-Kat. Second, the DNA fragment encoding the Phup-SOD-Thup expression cassette was PCR-amplified from the Pgap-Kat-Phup-SOD-Thup expression cassette (Zuo et al., 2014) and cloned into pBES2 via *FseI* and *BstBI* to construct pB-SK. All the restriction enzymes used in this study were purchased from NEB, Ipswich, MA, USA.

Successful cloning was confirmed by nucleotide sequencing. Each sub-cloned vector was introduced into *E. coli* via heat-shock and the $CaCl_2$ method and introduced into *B. bifidum* BGN4 by electroporation with the Gene Pulser Xcell Microbial Electroporation System (Bio-Rad, Hercules, CA, USA). The positive colonies were isolated, and the plasmids were purified and identified by restriction and sequence analyses.

Table 5.2 Primers for amplifying the SOD and catalase genes

Primer name	Sequences of primer Forward (5’ →3’)	Reverse (5’ →3’)	Product	Product information
primer Kat	TggcgcgccTGATGA TTCGAGAC	TggccggccTTAATCA CTGATAATAT	Pgap+Kat	gap gene (glyceraldehyde 3-phosphate dehydrogenase) promoter in *B. longum* + catalase gene in *Lactobacillus plantarum*
primer SOD	TggccggccTTGTCCG TTTTTGTCCAT	AggcgccCTGAACTAG TCCGGAAT	Phup+SOD+Thup	hup gene (histone-like protein HU) promoter in *B. longum* + SOD gene in *Streptococcus thermophilus* + hup gene terminator in *B. longum*

5.2.3 DNA manipulation

Plasmid DNA was extracted from an *Escherichia coli* DH5α transformant harboring pBES2 or each sub-cloned vector with a Plasmid Purification Mini Kit (Nucleogen, Gyeonggi-do, South Korea) and methylated *in vitro* by GpC (M.CviPI) methyltransferase (NEB, Ipswich, MA, USA). To identify the plasmid from the *B. bifidum* BGN4 transformants, plasmid DNA was extracted with a Plasmid Purification Mini Kit (Nucleogen) following an initial lysis step. Cells were resuspended in lysis buffer supplemented with lysozyme (20 mg / ml) and incubated at 37℃ for 1 h. The extracted DNA was identified by sequencing and comparing the restriction patterns with the original plasmid DNA derived from *E. coli*.

5.2.4 Measurement of the catalase activity

A colorimetric assay of catalase was performed as described by Asru K. Sinha (Sinha, 1972).

First, dichromate/acetic acid reagent was prepared by mixing a 5% solution of $K_2Cr_2O_7$ with glacial acetic acid (1:3, v/v). Then, bacterial cells grown in MRS media supplemented with 10 μM or 30 μM hematin (final concentration) were harvested and resuspended in 1 ml of 0.01 M phosphate buffer at various pH (pH 5.0 – 7.5) and mixed

with the assay mixture containing 4 ml of 0.8 mmol H_2O_2 and 5 ml of the above buffer. The reaction was run between 25 and 40℃. Then, 1 ml of the reaction mixture was withdrawn and added to 2 ml of dichromate/acetic acid reagent. It was then heated in a boiling water bath for 10 min, cooled at room temperature and measured at 570 nm with a spectrophotometer (Model 680 Microplate reader; Bio-Rad, Hercules, CA, USA).

5.2.5 Transformant selection under oxidative stress

BGN4-SK and BGN4 were inoculated into MRS media containing 0.05% L-cysteine·HCl and 30 μM hematin (final concentration), respectively, and cultured for 15 h. 1 ml of the BGN4 culture was put into a 2 ml tube and centrifuged at 10,000 ×g for 3 min. The pellet was resuspended in 1 ml of 0.01 M phosphate buffer (pH 6.0) and added to 1 ml of BGN4-SK diluted to 10^{-6} with the same buffer. Next, 30 μM Hematin (final concentration) and 4 mM H_2O_2 (final concentration) were added to the bacterial mixture (BGN4 and BGN4-SK), and the reaction mixture was incubated at 30℃ for 1 h. The reacted bacteria were centrifuged (10,000 ×g for 3 min at room temperature), and the harvested pellet was washed with the above buffer. The washed bifidobacterial cells were resuspended in 2 ml of the same buffer. Again, 30 μM Hematin (final concentration) and 4 mM H_2O_2 (final concentration) were added to the resuspended cells,

and the reaction mixture was incubated at 30℃ for 1 h. After the centrifugation (10,000 ×g for 3 min at room temperature) and washing with the same buffer, the bacterial cells reacted with H_2O_2 were then plated on MRS agar medium containing 30 μM Hematin (final concentration). All plates were incubated at 37℃ for at least 72 h. BGN4-SK colonies were selected by the bubble reaction between the catalase and H_2O_2. A drop of 30% H_2O_2 was applied with a syringe to the edge of each colony. BGN4-SK colonies which express catalase evolved bubbles of oxygen and were immediately streaked on a new MRS plate.

5.3 Results

5.3.1 Cloning of the SOD and catalase genes, and heterologous expression in *B. bifidum* BGN4

Given the reports that SOD and catalase are deficient or absent in *Bifidobacterium* (Shimamura et al., 1992) and the survival rate of *Bifidobacterium* in which SOD and catalase were heterologously expressed was drastically increased under oxidative stress (He et al., 2012; Zuo et al., 2014), the SOD and catalase genes were intended to be used as a food-grade selection marker to replace the antibiotic resistance genes in this study. To try this, a *Bifidobacterium* strain that strongly expresses SOD and catalase was necessary.

The SOD and catalase genes to be used in this study should be obtained from GRAS (Generally recognized as safe) microorganisms to be used in the construction of a food-grade vector. I reviewed a study by Fanglei Zuo *et al.* (Zuo et al., 2014) in which SOD and catalase derived from lactic acid bacteria were highly expressed in bifidobacteria with combinations of promoters and a terminator from *B. longum*, and the recombinant had a survival rate up to 10^4 times higher than that of the wild-type in the oxidative stress caused by

H_2O_2. The Kat-SOD expression cassette used in their study was thankfully provided by Fanglei Zuo *et al.* after contacting them.

B. bifidum BGN4 was selected as a transformation host in which SOD and catalase are expressed heterogeneously because this strain is genetically accessible among bifidobacteria (Park et al., 2018) and is relatively oxygen sensitive (data not shown). The SOD and catalase genes were amplified from the Kat-SOD expression cassette and subcloned into pBES2 with combinations of bifidobacterial promoters and a terminator and transformed into BGN4 as the host. Information about the genes, bifidobacterial promoters and the terminator used in this study is listed in Table 5.2.

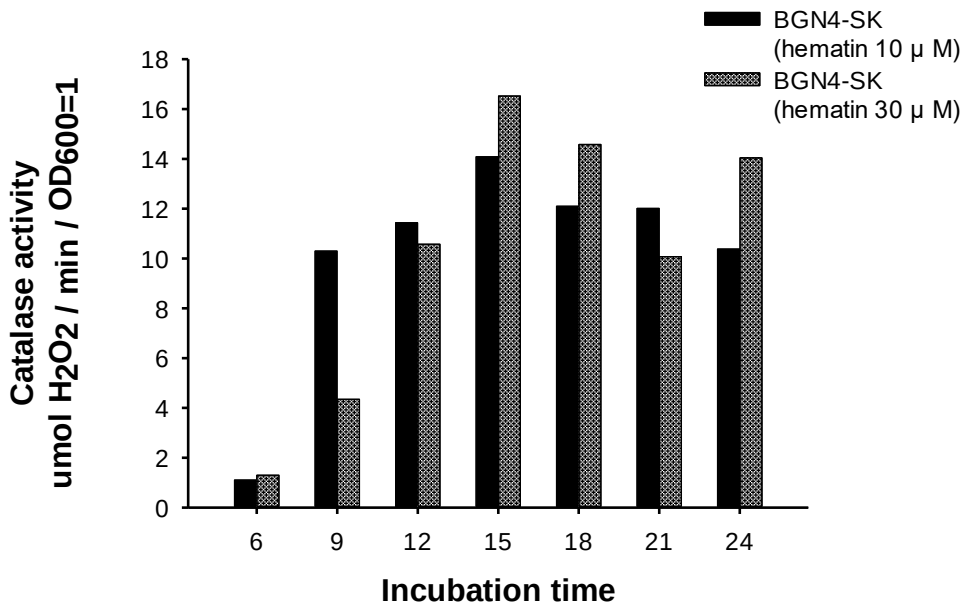
5.3.2 Optimization of the catalase activation conditions

Expression of the catalase in the recombinant BGN4 (BGN4-SK) was confirmed by the bubble reaction with H_2O_2 (data not shown). Because the Kat-SOD expression cassette provided by Fanglei Zuo *et al.* extremely raised the survival rate of a recombinant under oxidative stress induced by H_2O_2 rather than by O_2 or methyl viologen (Zuo et al., 2014), the BGN4-SK can be more clearly identified with the wild-type BGN4 under H_2O_2 stress. Therefore, it was necessary to first find the optimized conditions (such as the concentration of hematin added to the medium, incubation time, pH and temperature) at which the catalase can be expressed at the highest level in the

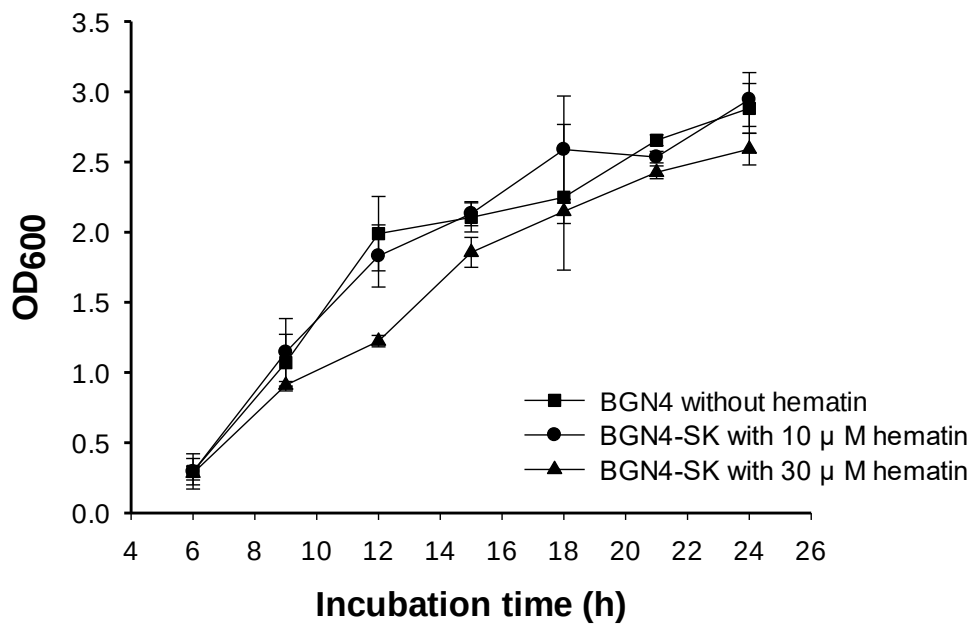
BGN4-SK. The catalase activity was measured by colorimetric assay designed by Asru K. Sinha (Sinha, 1972) and was expressed as the amount of H_2O_2 decomposed per min.

Because the catalase used in this study is a heme-dependent catalase, in order for the catalase to become active, hematin should be present in the medium in which the recombinant is cultured. In previous studies, 10 or 30 μM hematin (final concentration) was added to the culture medium (Abriouel et al., 2004; An et al., 2011; He et al., 2012; Zuo et al., 2014); thus, I decided to determine at which hematin concentration the catalase activity is stronger. As a result, BGN4-SK showed the highest catalase activity when cultured in MRS media supplemented with 30 μM hematin (final concentration) for 15 h (Fig. 5.1.A). Incidentally, the growth rate of the BGN4-SK in MRS media containing hematin was compared with the growth rate of the wild-type BGN4 grown in MRS media without hematin, and no specific change was observed in the growth curve, although the bacterial growth rate was slightly inhibited at 30 μM hematin (Fig. 5.1.B). Meanwhile, the optimal temperature and pH at which the catalase in BGN4-SK was most highly expressed were also investigated and the results are shown in Fig. 5.1.C, 5.1.D. The greatest catalase activity of BGN4-SK was observed at 30℃ and pH 6.0.

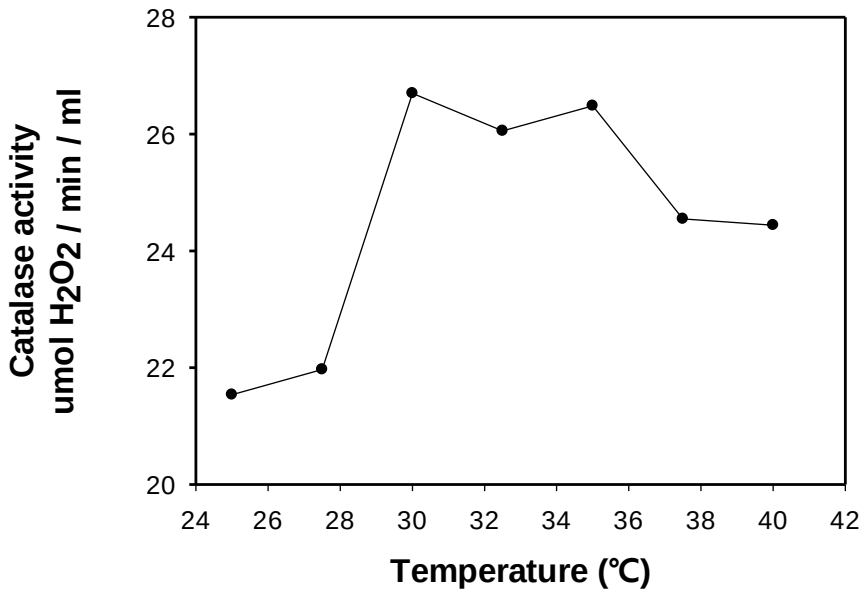
A


BGN4-SK
(hematin 10 μ M)
BGN4-SK
(hematin 30 μ M)
Catalase activity
umol H2O2 / min / OD600=1
0
2
4
6
8
10
12
14
16
18
6
9
12
15
18
21
24
Incubation time

B


OD600
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4
6
8
10
12
14
16
18
20
22
24
26
Incubation time (h)
BGN4 without hematin
BGN4-SK with 10 μ M hematin
BGN4-SK with 30 μ M hematin

C


Catalase activity
umol H2O2 / min / ml
28
26
24
22
20
24
26
28
30
32
34
36
38
40
42
Temperature (℃)

D

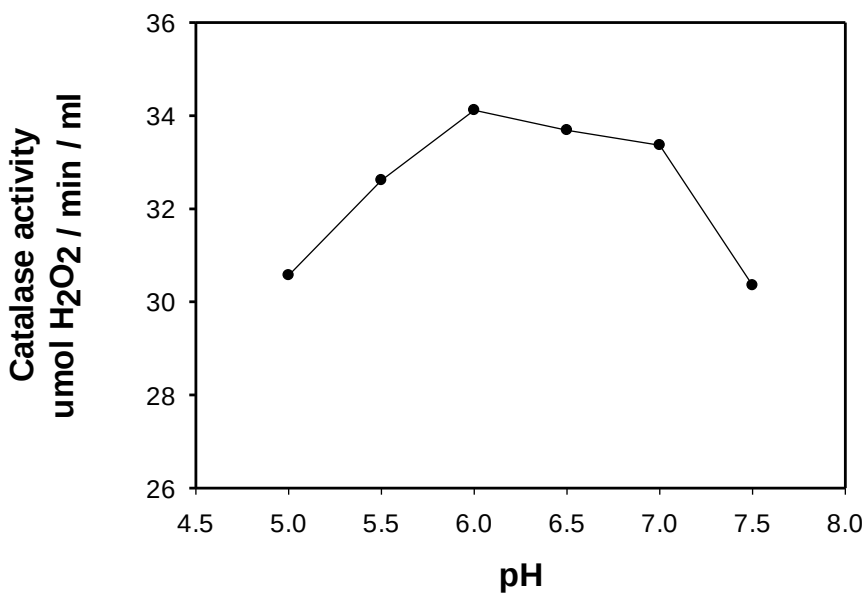

Catalase activity
umol H2O2 / min / ml
36
34
32
30
28
26
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
pH

Fig. 5.1. Optimal conditions at which the catalase can be expressed at the highest level in the BGN4-SK

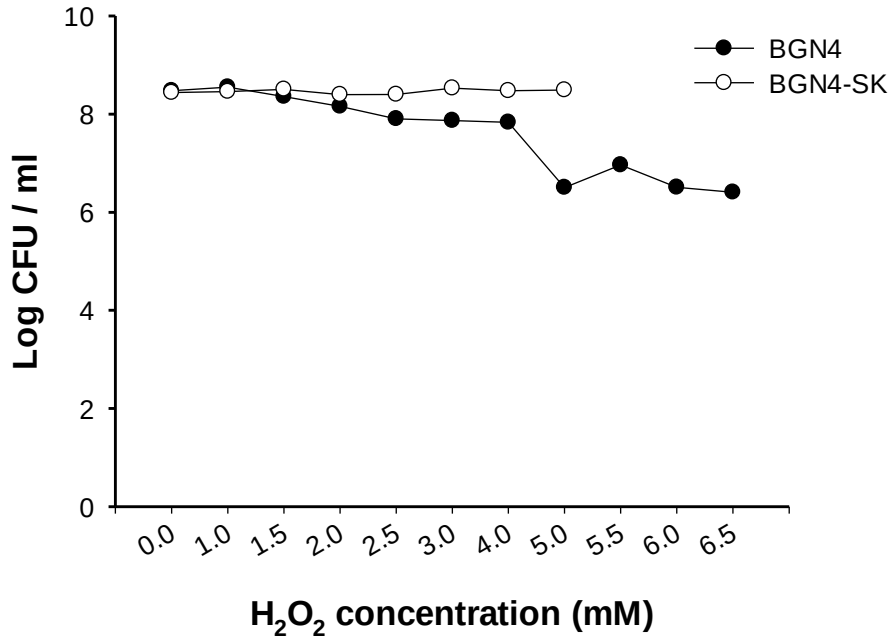
A combination of the concentration of hematin added to the medium and the incubation time (A), temperature (C) and pH (D) were investigated. Incidentally, the growth rate of BGN4-SK in MRS media containing hematin was compared with the growth rate of wild-type BGN4 grown in MRS media without hematin (B). Catalase activity was measured by colorimetric assay using the principle of obtaining chromic acetate by reacting H_2O_2 with dichromate in acetic acid and was expressed as the amount of H_2O_2 decomposed per min.

5.3.3 Optimal H_2O_2 stress for transformant selection

The optimal conditions for the catalase activity, which is crucial to H_2O_2 decomposition, were investigated before. Next, it was necessary to find the optimal H_2O_2 stress that significantly differentiates the survival rate between the wild-type BGN4 and BGN4-SK. Approximately 10^8 CFU/ml of BGN4 or BGN4-SK, cultured for 15 h in MRS media containing 30 μM of hematin (final concentration), were resuspended in 0.01 M phosphate buffer (pH 6.0) and reacted for 1 h at 30℃ in H_2O_2 concentrations ranging from 1.0 to 6.5 mM (final concentration). As a result, as the H_2O_2 concentration increased, the BGN4-SK still maintained viable cells of 10^8 CFU/ml whereas the viable cells of BGN4 decreased to 10^6 CFU/ml (Fig. 5.2.A). However, there was a limitation in that the viable count of the wild-type BGN4 did not decrease drastically enough to replace the antibiotic resistance marker. Although it was possible to increase H_2O_2 concentration even more, it was reported that H_2O_2 causes DNA degradation (Collins, 1999; Sadhu et al., 2018); therefore, I decided to increase the number of reactions at a mild H_2O_2 concentration rather than increase the H_2O_2 concentration. Notably, when reacted at a relatively mild H_2O_2 concentration of 4 mM, the viable count of BGN4-SK still remained at 10^8 CFU/ml while that of BGN4 rapidly decreased below 10^4 CFU/ml from the second reaction (Fig. 5.2.B). It is suggested that SOD and catalase are likely to replace the antibiotic resistance marker because of leading the

markedly different survival rates of BGN4 and BGN4-SK in the optimized conditions.

A


10
8
6
4
2
0
Log CFU / ml
BGN4
BGN4-SK
0.0
1.0
1.5
2.0
2.5
3.0
4.0
5.0
5.5
6.0
6.5
H2O2 concentration (mM)

B

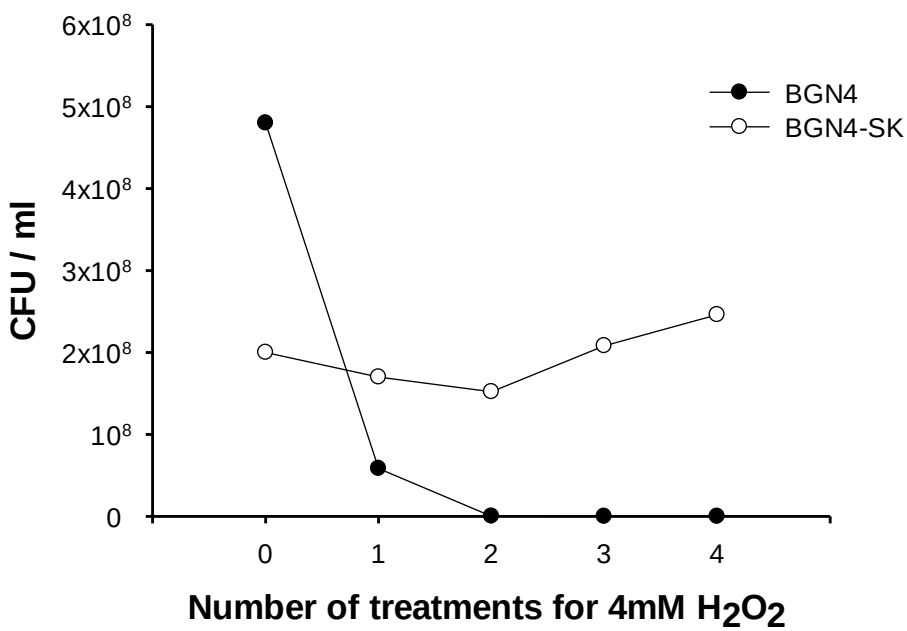

6x10^8
5x10^8
4x10^8
3x10^8
2x10^8
10^8
0
CFU / ml
BGN4
BGN4-SK
0
1
2
3
4
Number of treatments for 4mM H2O2

Fig. 5.2 Optimal H_2O_2 stress for transformant selection

Changes in survival rate of BGN4 and BGN4-SK by using various concentrations of H_2O_2 (A) or by increasing the number of reactions at a mild H_2O_2 concentration (B) were investigated.

5.3.4 Possibility as a selection marker for a food-grade vector

Assuming that the transformation efficiency is low, 10^2 CFU of BGN4-SK were mixed with 10^8 CFU of wild-type BGN4, and then, it was examined whether BGN4-SK is distinguishable from BGN4 under the H_2O_2 stress set in this study. The selection pressure by H_2O_2 was given by repeating twice the exposure to 4 mM H_2O_2 in 0.01 M phosphate buffer (pH 6.0) for 1 h at 30℃. Notably, most of the cells were killed and only 34 colonies remained. Bubbles emerged from two colonies as a result of dropping H_2O_2 onto the edge of each colony (Fig. 5.3) After the bubble-forming colonies were inoculated into the MRS medium, the plasmid was extracted and sequenced. The sequencing results were consistent with pB-SK, and there were no mutations in the nucleotide sequence. This means that the selection pressure induced by H_2O_2 is suitable for identifying a few recombinant BGN4 in the majority of wild-type BGN4 and that there is no plasmid DNA damage by the H_2O_2 stress set in this study.

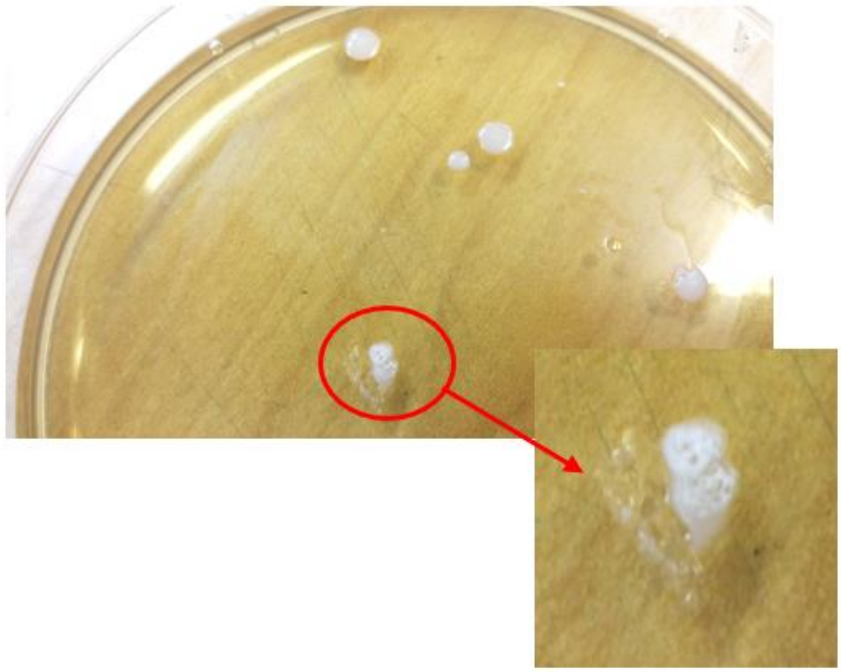


Fig. 5.3 BGN4-SK colony forming bubble upon contact with H_2O_2

When the bacterial mixture of 10^2 CFU of BGN4-SK and 10^8 CFU of wild-type BGN4 was treated with the set H_2O_2 stress, most of the cells were dead, and only 34 colonies remained, of which 2 colonies were identified as BGN4-SK. The bubbling colony in this photograph was one of two BGN4-SK colonies that survived the H_2O_2 stress.

5.4 Discussion

Reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot -}$), and hydroxyl radical ($^{\cdot}OH$) are produced as by-products of cellular metabolism, mainly in the mitochondria. When ROS production overwhelms the antioxidant capacity, damage to cellular protein, DNA, lipid and membranes may ensue (Storz and Imlayt, 1999; Thannickal and Fanburg, 2000). SOD and catalase are typical antioxidant enzymes; thus, they are important components of the antioxidant defense system in cells against oxygen (Shimamura et al., 1992). However, these enzyme activities are deficient or absent in almost all obligate anaerobic bacteria including bifidobacteria, and in reality, neither sod nor catalase homologs are present in the genome of bifidobacteria (He et al., 2012; Zuo et al., 2014). Recently, there have been studies in which SOD and catalase were heterogeneously expressed in bifidobacteria, and the survival rate of the recombinant bifidobacteria expressing them was increased up to 10^4 – 10^5 fold compared to the wild-type bifidobacteria under oxidative stress. Based on these results, the present study evaluated the possibility of the SOD and catalase genes as a selection marker for the development of a food-grade vector for bifidobacteria which has not been constructed to date.

The Kat-SOD expression cassette used in this study was thankfully

provided by Fanglei Zuo *et al.* after contacting them. The catalase gene was derived from *Lactobacillus plantarum*, and the gap gene (glyceraldehyde 3-phosphate dehydrogenase) promoter in *B. longum* was incorporated upstream of the catalase gene. The SOD gene was derived from *Streptococcus thermophilus*, and the hup gene (histone-like protein HU) promoter and terminator in *B. longum* were introduced upstream and downstream of the SOD gene, respectively. In other words, the components of the Kat-SOD expression cassette, used as substitutes for antibiotic resistance markers in this study, were all derived from food-grade microorganisms. Using this expression cassette, BGN4-SK, which is a recombinant *B. bifidum* BGN4 heterologously expressing SOD and catalase, was developed.

The Kat-SOD expression cassette provided by Fanglei Zuo *et al.* drastically raised the survival rate of a recombinant under oxidative stress induced by H_2O_2 rather than by O_2 or methyl viologen (Zuo et al., 2014); thus, it was decided to use H_2O_2 stress to distinguish the BGN4-SK from the wild-type BGN4. Because catalase has a crucial role in the decomposition of H_2O_2, it was necessary to first find the optimal conditions at which the catalase can be expressed at the greatest level in the BGN4-SK. The concentration of hematin added to the medium, incubation time, pH and temperature, which are factors that maximize catalase activity in BGN4-SK, were established through the optimization experiments (Fig. 5.1). Next,

the optimal H_2O_2 stress was investigated to clearly distinguish the survival rates of the wild-type BGN4 and BGN4-SK. As a result, when reacted twice for 1 h at a relatively mild H_2O_2 concentration of 4 mM, the viable count of BGN4-SK still remained almost unchanged while that of BGN4 extremely decreased by more than 99% (Fig. 5.2). This result implies that SOD and catalase can likely replace the antibiotic resistance marker because of leading the drastically different survival rates of BGN4 and BGN4-SK in the optimized conditions. In reality, when a bacterial mixture of 10^2 CFU of BGN4-SK and 10^8 CFU of wild-type BGN4 was treated with the set H_2O_2 stress, most of the cells were dead, and only 34 colonies remained, of which 2 colonies were identified as BGN4-SK. Collectively, the results suggest that the selection pressure induced by H_2O_2, which I found, are suitable for identifying a few recombinants BGN4 in the majority of the wild-type BGN4.

Because it was identified that SOD and catalase can possibly act as a selection marker, I would like to explain the scheme of the future development of a food-grade vector for bifidobacteria. The first step should be to confirm whether selection of BGN4-SK is possible under the set H_2O_2 stress during the electroporation process. This experiment is currently underway in my laboratory and will proceed to the next step if it is successful. Because two antibiotic resistance genes remain in BGN4-SK, one of them should be removed by PCR, restriction-enzyme digestion and ligation, and then, the target genes

are subcloned into the plasmid using the other antibiotic resistance marker when selecting recombinants in *E. coli*. After that, the remaining antibiotic resistance gene from the plasmid is removed by PCR, restriction-enzyme digestion and ligation. The ligated vector is immediately transformed into *Bifidobacterium*, and the recombinant bifidobacteria are selected between the wild-type bifidobacteria under the H_2O_2 stress set in this study. At this time, the electroporation efficiency will be a key factor. Although the electroporation efficiency when the ligated vector was immediately transformed into BGN4 was reduced to 1/20 of that of the untreated vector in my previous study (data not shown), the electroporation conditions were optimized to raise the electroporation efficiency of BGN4 to 10^5 CFU/μg DNA, which would offset this disadvantage (Park et al., 2018).

However, there are limitations in using the SOD and catalase genes as a selection marker for bifidobacteria. Because the reproducibility is not high yet, only three out of 10 trials that selected a few BGN4-SK among the majority of wild-type BGN4 were successful. When treating 10^8 CFU of wild-type BGN4 with H_2O_2 stress, the final number of colonies was sometimes less than 100 CFU and sometimes more than 10,000 CFU. The experiments are not clearly controlled because no definite cause has been found yet.

Nevertheless, it is meaningful that this study searched for a candidate selection marker that can advance the development of a

food-grade vector for bifidobacteria. Because most bifidobacteria do not express SOD and catalase, SOD and catalase can be studied as a selection marker with a broad host-spectrum in bifidobacteria, if the selection conditions by H_2O_2 are optimized for each bifidobacterial strain.

Chapter 6.

Conclusion

The aim of this study was to obtain a new recombinant bifidobacteria that highly expresses β-galactosidase by using molecular genetic techniques and utilize it *in vitro* lactose hydrolysis and *in vivo* lactose-intolerant animal experiment.

Prior to the development of the β-galactosidase over-expressing bifidobacteria, a preliminary study was conducted to improve the electroporation-mediated transformation efficiency of bifidobacteria to a reproducibly high level in order to effectively introduce β-galactosidase into bifidobacteria. As a result, when the cell wall weakening agent (NaCl) and cell membrane permeabilizing agent (Ethanol) were applied and the electrical parameter was adjusted from 2.5 kV to 3 kV, the electroporation-mediated transformation efficiency of *B. bifidum* BGN4, which was a transformation host in my study, drastically and consistently increased from 10^3 to 10^5 CFU / μg DNA. Because the method was extensively applied to other *Bifidobacterium* species, this should allow for the advanced genetic manipulation of the various *Bifidobacterium* species in future studies.

Returning to main study, a β-galactosidase over-expressing bifidobacteria strain was developed by using a heterologous gene from *B. longum* RD47 which is one of the bifidobacterial strains with the greatest level of β-galactosidase activity. Among the recombinant bifidobacteria, BGN4-G1 has the highest β-galactosidase activity, for which the hydrolytic activity was 2.5 to 4.2 times higher than that of BGN4 and 4.3 to 9.6 times higher than that

of the wild-type RD47. The β-galactosidase level of BGN4-G1 was remarkably superior to that of any of the other 35 lactic acid bacteria. When commercial whole milk and BGN4-G1 were reacted, BGN4-G1 hydrolyzed 50% of the lactose in milk at 63 h and finally 61% at 93 h during fermentation. This figure is about twice the lactose removal rate of conventional fermented milk. In the case of the reaction of commercial whole milk and a crude enzyme extract with 50 μg of protein from BGN4-G1, the β-galactosidase of BGN4+G1 hydrolyzed half of the lactose in whole milk in 2 h, and it is expected to have a greater effect if the amount of crude enzyme extract to be used for the reaction is increased. Therefore, it is believed that the purification of β-galactosidase from BGN4-G1 by attaching a his-tag to the gene will have industrial significance, such as using it in the production of lactose-free milk or fermented milk.

Probiotics administration has received attention as one of the LI treatments. However, because of the contradictory results of previous studies, no clear conclusion has been drawn as to whether probiotics have an effect on LI. I carefully thought that the conflicting results may be due to the different β-galactosidase activities of the probiotics strains used in each study and devised experiments using bifidobacteria, which have a relatively high β-galactosidase activity among the probiotics, with different β-galactosidase levels to confirm the hypothesis. Therefore, the present work evaluated *in vivo* the effect of the β-galactosidase activity of bifidobacteria on

LI alleviation by administering β-galactosidase reduced bifidobacteria, wild-type bifidobacteria and β-galactosidase over-expressing bifidobacteria to post-weaning Balb/c mice which have been shown to be the LI model mice. BGN4-G1 was used as a β-galactosidase over-expressing bifidobacteria strain and in the case of a β-galactosidase reduced BGN4, a BGN4 mutant (BGN4-bgr) showing approximately 3.2 times lower enzyme activity than that of the wild-type was developed by randomized mutagenesis. As a result, the LI alleviation effect in the BGN4-G1 administration group was greater than that in the lactase-treated group and even reached a LI-uninduced level. BGN4 administration slightly alleviated LI symptoms compared to BGN4-bgr administration which had no significant effects. This suggests that the LI alleviation effect is improved as the β-galactosidase activity of the bifidobacteria is increased.

To explore the mechanisms for the demonstrated better lactose tolerance of the bifidobacteria highly expressing β-galactosidase, the lactose metabolism level in the small intestine was first monitored. Because elevated levels of plasma glucose after 30 min post-lactose and β-galactosidase activities of the small intestine in the bifidobacteria-fed groups were not significantly different compared to the untreated group, the mechanism of LI alleviation as the increases of β-galactosidase activity from the bifidobacteria is not

much related to the lactose metabolism in the small intestine by the β-galactosidase activities of the small intestine itself and the bacteria present there. Therefore, it was necessary to explore the mechanisms of LI alleviation from other aspects. Previous studies have reported that LI is also associated with intestinal flora. Based on this viewpoint, a gut microbiome analysis was performed from the feces of the mice fed BGN4-bgr, BGN4, BGN4-G1 or NS for two weeks to investigate the LI alleviation mechanism. Unfortunately, the gut microbiome analysis did not clearly reveal the mechanism of the better lactose tolerance by the β-galactosidase level in the bifidobacteria. However, some meaningful results were derived as follows: 1) The proportion of several microorganisms associated with intestinal health tended to be differentiated between the *Bifidobacterium*-fed groups and the untreated group, 2) The bifidobacterial proportion in the BGN4-G1 group tended to be increased compared to the other *Bifidobacterium*-fed groups, 3) The balance of SCFAs and lactate was differentiated between the *Bifidobacterium*-fed groups and the untreated group, 4) The rates for the change in SCFAs and lactate were grouped in a similar pattern in the BGN4-G1 and BGN4 groups except for the BGN4-bgr group.

Because the BGN4-G1 exhibited a significant effect on LI alleviation, it is expected that BGN4-G1 can be reproduced by using a food-grade vector for bifidobacteria, and it will be utilized as an LI treatment strain. I decided to construct a food-grade vector for

bifidobacteria and noted that bifidobacteria are obligate anaerobic bacteria, so they are deficient or absent in antioxidant enzyme activities such as SOD and catalase. The present experiment evaluated the possibility of SOD and catalase genes as selection markers for the development of food-grade vector for bifidobacteria that has not been constructed to date.

First, BGN4-SK, a recombinant *Bifidobacterium* heterologously expressing food-grade SOD and catalase, was developed. In order to use H_2O_2 stress to distinguish BGN4-SK from wild-type BGN4, the concentration of hematin added to the medium, incubation time, pH and temperature which are factors that maximize catalase activity in BGN4-SK were established through the optimization experiment. Next, optimal H_2O_2 stress was investigated to clearly distinguish the survival rates of wild-type BGN4 and BGN4-SK. As a result, when reacted twice for 1 h at a relatively mild H_2O_2 concentration of 4 mM, the viable count of BGN4-SK still remained almost unchanged, while that of BGN4 extremely decreased more than 99%. It implies that SOD and catalase are likely to replace antibiotic resistance marker because of leading the drastically different survival rates of BGN4 and BGN4-SK in the optimized conditions. In reality, when the bacterial mixture of 10^2 CFU of BGN4-SK and 10^8 CFU of wild-type BGN4 was treated with the set H_2O_2 stress, most of the cells were dead and only 34 colonies remained, of which 2 colonies were identified as BGN4-SK. Collectively, the results suggest that the

selection pressure induced by H_2O_2 are suitable for identifying a few recombinants BGN4 in the majority of wild-type BGN4. It is meaningful that this experiment searched for a candidate of selection marker that can advance the development of a food-grade vector for bifidobacteria. Because most bifidobacteria do not express SOD and catalase, SOD and catalase can be studied as a selection marker with a broad host-spectrum in bifidobacteria, if the selection conditions by H_2O_2 are optimized for each bifidobacterial strain.

References

Abriouel, H., A. Herrmann, J. Stärke, N.M. Yousif, A. Wijaya, B. Tauscher, W. Holzapfel, and C.M. Franz. 2004. Cloning and heterologous expression of hematin-dependent catalase produced by *Lactobacillus plantarum* CNRZ 1228. *Appl. Environ. Microbiol.* 70:603-606.

Almeida, C.C., S.L.S. Lorena, C.R. Pavan, H.M.I. Akasaka, and M.A. Mesquita. 2012. Beneficial effects of long-term consumption of a probiotic combination of *Lactobacillus casei* Shirota and *Bifidobacterium breve* Yakult may persist after suspension of therapy in lactose-intolerant patients. *Nutr. Clin. Pract.* 27:247-251.

Alvarez-Sieiro, P., M.C. Martin, B. Redruello, B. Del Rio, V. Ladero, B.A. Palanski, C. Khosla, M. Fernandez, and M.A. Alvarez. 2014. Generation of food-grade recombinant *Lactobacillus casei* delivering *Myxococcus xanthus* prolyl endopeptidase. *Appl. Microbiol. Biotechnol.* 98:6689-6700.

An, H., Z. Zhai, S. Yin, Y. Luo, B. Han, and Y. Hao. 2011. Coexpression of the superoxide dismutase and the catalase provides remarkable oxidative stress resistance in *Lactobacillus rhamnosus*. *J. Agric. Food Chem.* 59:3851-3856.

Aoki, R., S. Tsuchida, Y. Arai, K. Ohno, T. Nishijima, T. Mawatari, Y. Mikami, and K. Ushida. 2016. Effect of *Bifidobacterium animalis* subsp. *lactis* GCL2505 on the physiological function of intestine in a rat model. *Food Sci. Nutr.* 4:782-790.

Apajalahti, J.H., H. Kettunen, A. Kettunen, W.E. Holben, P.H. Nurminen, N. Rautonen, and M. Mutanen. 2002. Culture-independent microbial community analysis reveals that inulin in the diet primarily affects previously unknown bacteria in the mouse cecum. *Appl. Environ. Microbiol.* 68:4986-4995.

Argnani, A., R.J. Leer, N. van Luijk, and P.H. Pouwels. 1996. A convenient and reproducible method to genetically transform bacteria of the genus *Bifidobacterium*. *Microbiology*. 142:109-114.

Assad-García, J., M. Bonnin-Jusserand, D. Garmyn, J. Guzzo, H. Alexandre, and C. Grandvalet. 2008. An improved protocol for electroporation of *Oenococcus oeni* ATCC BAA-1163 using ethanol as immediate membrane fluidizing agent. *Lett. Appl. Microbiol.* 47:333-338.

Aune, T.E.V., and F.L. Aachmann. 2010. Methodologies to increase the transformation efficiencies and the range of bacteria that can be transformed. *Appl. Microbiol. Biotechnol.* 85:1301-1313.

Barry, J.A., and K. Gawrisch. 1994. Direct NMR evidence for ethanol binding to the lipid-water interface of phospholipid bilayers. *Biochemistry*. 33:8082-8088.

Baskaran, S., H.J. Ahn, and L.R. Lynd. 1995. Investigation of the ethanol tolerance of *Clostridium thermosaccharolyticum* in continuous culture. *Biotechnol. Prog.* 11:276-281.

Beards, E., K. Tuohy, and G. Gibson. 2010. Bacterial, SCFA and gas profiles of a range of food ingredients following in vitro fermentation by human colonic microbiota. *Anaerobe*. 16:420–425.

Becerra, M., S.D. Prado, E. Cerdán, and M.G. Siso. 2001. Heterologous *Kluyveromyces lactis* β–galactosidase secretion by *Saccharomyces cerevisiae* super–secreting mutants. *Biotechnol. Lett.* 23:33–40.

Benavente, R., B. Pessela, J. Curiel, B. de las Rivas, R. Muñoz, J. Guisán, J. Mancheño, A. Cardelle–Cobas, A. Ruiz–Matute, and N. Corzo. 2015. Improving properties of a novel β–galactosidase from *Lactobacillus plantarum* by covalent immobilization. *Molecules*. 20:7874–7889.

Bermúdez–Humarán, L.G., P. Kharrat, J.–M. Chatel, and P. Langella. 2011. Lactococci and lactobacilli as mucosal delivery vectors for therapeutic proteins and DNA vaccines. *Microb. Cell Fact.* 10(Suppl 1):S4

Bidart, G.N., J. Rodríguez–Díaz, G. Pérez–Martínez, and M.J. Yebra. 2018. The lactose operon from *Lactobacillus casei* is involved in the transport and metabolism of the human milk oligosaccharide core–2 n–acetyllactosamine. *Sci. Rep.* 8:7152.

Blaut, M., M. Collins, G. Welling, J. Dore, J. Van Loo, and W. De Vos.

2002. Molecular biological methods for studying the gut microbiota: the EU human gut flora project. *Br. J. Nutr.* 87:S203-S211.

Bottacini, F., R. Morrissey, R.J. Roberts, K. James, J. van Breen, M. Egan, J. Lambert, K. van Limpt, J. Knol, and M.O.C. Motherway. 2017. Comparative genome and methylome analysis reveals restriction/modification system diversity in the gut commensal *Bifidobacterium breve*. *Nucleic Acids Res.* 46:1860-1877.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.

Campbell, J.M., G.C. Fahey Jr, and B.W. Wolf. 1997. Selected indigestible oligosaccharides affect large bowel mass, cecal and fecal short-chain fatty acids, pH and microflora in rats. *J. Nutr*. 127:130-136.

Cano-Garrido, O., J. Seras-Franzoso, and E. Garcia-Fruitos. 2015. Lactic acid bacteria: reviewing the potential of a promising delivery live vector for biomedical purposes. *Microb. Cell Fact*. 14:137.

Carneiro, S., E.C. Ferreira, and I. Rocha. 2013. Metabolic responses to recombinant bioprocesses in *Escherichia coli*. *J. Biotechnol.* 164:396-408.

Ceroni, F., A. Boo, S. Furini, T.E. Gorochowski, O. Borkowski, Y.N. Ladak, A.R. Awan, C. Gilbert, G.-B. Stan, and T. Ellis. 2018.

Burden-driven feedback control of gene expression. *Nat. Methods*. 15:387.

Ceroni, F., R. Algar, G.-B. Stan, and T. Ellis. 2015. Quantifying cellular capacity identifies gene expression designs with reduced burden. *Nat. Methods*. 12:415.

Chan, V., L.F. Dreolini, K.A. Flintoff, S.J. Lloyd, and A.A. Mattenley. 2002. The effects of glycerol, glucose, galactose, lactose and glucose with galactose on the induction of β-galactosidase in *Escherichia coli*. *J. Exp. Microbiol. Immunol.* 2:130-137.

Chanalia, P., D. Gandhi, P. Attri, and S. Dhanda. 2018. Purification and characterization of β-galactosidase from probiotic *Pediococcus acidilactici* and its use in milk lactose hydrolysis and galactooligosaccharide synthesis. *Bioorg. Chem.* 77:176-189.

Chen, J., N. Chia, K.R. Kalari, J.Z. Yao, M. Novotna, M.M.P. Soldan, D.H. Luckey, E.V. Marietta, P.R. Jeraldo, and X. Chen. 2016. Multiple sclerosis patients have a distinct gut microbiota compared to healthy controls. *Sci. Rep.* 6:28484.

Collins, A.R. 1999. Oxidative DNA damage, antioxidants, and cancer. *Bioessays*. 21:238-246.

Czermak, P., M. Ebrahimi, K. Grau, S. Netz, G. Sawatzki, and P.H. Pfromm. 2004. Membrane-assisted enzymatic production of galactosyl-oligosaccharides from lactose in a continuous process. *J. Membr. Sci.* 232:85-91.

Dahlqvist, A., N.-G. Asp, A. Burvall, and H. Rausing. 1977. Hydrolysis of lactose in milk and whey with minute amounts of lactase. *J. Dairy Res.* 44:541-548.

De Alcantara, P., L. Martim, C. Silva, S. Dietrich, and M. Buckeridge. 2006. Purification of a β-galactosidase from cotyledons of *Hymenaea courbaril* L.(Leguminosae). Enzyme properties and biological function. *Plant Physiol. Biochem.* 44:619-627.

De Vuyst, L., and F. Leroy. 2011. Cross-feeding between bifidobacteria and butyrate-producing colon bacteria explains bifdobacterial competitiveness, butyrate production, and gas production. *Int. J. Food Microbiol.* 149:73-80.

Dehkordi, N., D. Rao, A. Warren, and C. Chawan. 1995. Lactose malabsorption as influenced by chocolate milk, skim milk, sucrose, whole milk, and lactic cultures. *J. Am. Diet. Assoc.* 95:484-486.

Dekker, P.J., D. Koenders, and M.J. Bruins. 2019. Lactose-Free Dairy Products: Market Developments, Production, Nutrition and Health Benefits. *Nutrients*. 11:551.

Deng, Y., B. Misselwitz, N. Dai, and M. Fox. 2015. Lactose intolerance in adults: biological mechanism and dietary management. *Nutrients*. 7:8020-8035.

Di Stefano, M., G. Veneto, S. Malservisi, L. Cecchetti, L. Minguzzi, A. Strocchi, and G.R. Corazza. 2002. Lactose malabsorption and intolerance and peak bone mass. *Gastroenterology*.

122:1793-1799.

Ding, H., L. Zhou, Q. Zeng, Y. Yu, and B. Chen. 2018. Heterologous Expression of a Thermostable β-1, 3-Galactosidase and Its Potential in Synthesis of Galactooligosaccharides. *Mar. Drugs*. 16:415.

Dower, W.J., J.F. Miller, and C.W. Ragsdale. 1988. High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res.* 16:6127-6145.

During, M.J., R. Xu, D. Young, M.G. Kaplitt, R.S. Sherwin, and P. Leone. 1998. Peroral gene therapy of lactose intolerance using an adeno-associated virus vector. *Nat. Med.* 4:1131.

Eadala, P., S.B. Matthews, J.P. Waud, J.T. Green, and A.K. Campbell. 2011. Association of lactose sensitivity with inflammatory bowel disease-demonstrated by analysis of genetic polymorphism, breath gases and symptoms. *Aliment. Pharmacol. Ther*. 34:735-746.

Estrem, S.T., T. Gaal, W. Ross, and R.L. Gourse. 1998. Identification of an UP element consensus sequence for bacterial promoters. *Proc. Natl. Acad. Sci*. 95:9761-9766.

Fischer, W., W. Bauer, and M. Feigel. 1987. Analysis of the lipoteichoic-acid-like macroamphiphile from *Bifidobacterium bifidum* subspecies *pennsylvanicum* by one- and two-dimensional 1H- and 13C-NMR spectroscopy. *FEBS J.* 165:647-652.

Flint, H.J., S.H. Duncan, K.P. Scott, and P. Louis. 2015. Links between diet, gut microbiota composition and gut metabolism. *Proc. Nutr. Soc.* 74:13-22.

Fuller, R. 1991. Probiotics in human medicine. *Gut*. 32:439.

Gänzle, M.G., G. Haase, and P. Jelen. 2008. Lactose: crystallization, hydrolysis and value-added derivatives. *Int. Dairy J.* 18:685-694.

Gerber, S.D., and M. Solioz. 2007. Efficient transformation of *Lactococcus lactis* IL1403 and generation of knock-out mutants by homologous recombination. *J. Basic Microbiol.* 47:281-286.

Gibson, G.R., and M.B. Roberfroid. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J. Nutr*. 125:1401-1412.

Gold, R.S., M. Meagher, R. Hutkins, and T. Conway. 1992. Ethanol tolerance and carbohydrate metabolism in lactobacilli. *J. Ind. Microbiol*. 10:45-54.

Gory, L., M.-C. Montel, and M. Zagorec. 2001. Use of green fluorescent protein to monitor *Lactobacillus sakei* in fermented meat products. *FEMS Microbiol. Lett.* 194:127-133.

Guglielmetti, S., M. Karp, D. Mora, I. Tamagnini, and C. Parini. 2007. Molecular characterization of *Bifidobacterium longum* biovar *longum* NAL8 plasmids and construction of a novel replicon screening system. *Appl. Microbiol. Biotechnol.* 74:1053.

Hammes, W., K. Schleifer, and O. Kandler. 1973. Mode of action of glycine on the biosynthesis of peptidoglycan. *J. Bacteriol.* 116:1029-1053.

Han, Y.R., S.Y. Youn, G.E. Ji, and M.S. Park. 2014. Production of α- and β-galactosidases from *Bifidobacterium longum* subsp. *longum* RD47. *J. Microbiol. Biotechnol.* 24:675-682.

Hanahan, D., J. Jessee, and F.R. Bloom. 1991. Plasmid transformation of *Escherichia coli* and other bacteria. *Methods Enzymol.* 204:63-113.

Harju, M. 2004. Chromatographic and enzymatic removal of lactose from milk. *Int. Dairy Federation* 389:4-8.

Harju, M., H. Kallioinen, and O. Tossavainen. 2012. Lactose hydrolysis and other conversions in dairy products: Technological aspects. *Int. Dairy J.* 22:104-109.

Harrington, L., and J. Mayberry. 2008. A re-appraisal of lactose intolerance. *Int. J. Clin. Pract.* 62:1541-1546.

Hartke, A., A. Benachour, P. Boutibonnes, and Y. Auffray. 1996. Characterisation of a complex restriction/modification system detected in a *Bifidobacterium longum* strain. *Appl. Microbiol. Biotechnol.* 45:132-136.

Hashiba, H., R. Takiguchi, S. Ishii, and K. Aoyama. 1990. Transformation of *Lactobacillus helveticus* subsp. *jugurti* with plasmid pLHR by electroporation. *Agric. Biol. Chem.* 54:1537-1541.

He, J., K. Sakaguchi, and T. Suzuki. 2012. Acquired tolerance to oxidative stress in *Bifidobacterium longum* 105-A via expression of a catalase gene. *Appl. Environ. Microbiol.* 78:2988-2990.

He, M., and Y. Yang. 1999. Lactase deficiency and lactose intolerance. *Foreign Med. Sci*. 6:339-342.

He, T., M. Priebe, Y. Zhong, C. Huang, H. Harmsen, G. Raangs, J.M. Antoine, G. Welling, and R. Vonk. 2008. Effects of yogurt and bifidobacteria supplementation on the colonic microbiota in lactose-intolerant subjects. *J. Appl. Microbiol.* 104:595-604.

He, W., B. Lü, and C. Huang. 2005. Lactase and its gene regulation in BALB/c mice at different ages. *Sichuan Da Xue Xue Bao Yi Xue Ban*. 36:516-518.

Helmann, J.D. 1995. Compilation and analysus of *Bacillus Subtilis* σA-dependent promoter sequences: evidence for extended contact between RNA polymerse and upstream promoter DNA. *Nucleic Acids Res.* 23:2351-2360.

Heyman, M.B. 2006. Lactose intolerance in infants, children, and adolescents. *Pediatrics*. 118:1279-1286.

Hiele, M., Y. Ghoos, P. Rutgeerts, G. Vantrappen, H. Carchon, and E. Eggermont. 1988. $^{13}CO_2$ breath test using naturally ^{13}C-enriched lactose for detection of lactase deficiency in patients with gastrointestinal symptoms. *J. Clin. Lab. Med*. 112:193-200.

Hijova, E., and A. Chmelarova. 2007. Short chain fatty acids and colonic health. *Bratisl. Lek. Listy*. 108:354.

Holo, H., and I.F. Nes. 1989. High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. *Appl. Environ. Microbiol.* 55:3119-3123.

Hong, K.S., H.W. Kang, J.P. Im, G.E. Ji, S.G. Kim, H.C. Jung, I.S. Song, and J.S. Kim. 2009. Effect of probiotics on symptoms in Korean adults with irritable bowel syndrome. *Gut Liver*. 3:101.

Hsu, Chin-An., Roch-Chui. Yu, and Cheng-Chun Chou. 2006. Purification and characterization of a sodium-stimulated β-galactosidase from *Bifidobacterium longum* CCRC 15708. *World J. Microbiol. Biotechnol*. 22:355-361.

Hsu, CA., SL. Lee, and Chou CC. 2007. Enzymatic production of galactooligosaccharides by β-galactosidase from *Bifidobacterium longum* BCRC 15708. *J. Agric. Food Chem.* 55:2225-2230.

Hsu, C., R. Yu, and C. Chou. 2005. Production of β-galactosidase by bifidobacteria as influenced by various culture conditions. *Int. J. Food Microbiol.* 104:197-206.

Ibrahim, S.A., and D.J. O'Sullivan. 2000. Use of chemical mutagenesis for the isolation of food grade β-galactosidase overproducing mutants of bifidobacteria, lactobacilli and *Streptococcus thermophilus*. *J. Dairy Sci.* 83:923-930.

Ilyés, H., E. Fekete, L. Karaffa, É. Fekete, E. Sándor, A. Szentirmai, and C.P. Kubicek. 2004. CreA-mediated carbon catabolite repression of β-galactosidase formation in *Aspergillus nidulans* is growth rate dependent. *FEMS Microbiol. Lett.* 235:147-151.

Inchaurrondo, V., M. Flores, and C. Voget. 1998. Growth and β-galactosidase synthesis in aerobic chemostat cultures of *Kluyveromyces lactis*. *J. Ind. Microbiol. Biotechnol.* 20:291-298.

Ingram, L. 1986. Microbial tolerance to alcohols: role of the cell membrane. *Trends Biotechnol.* 4:40-44.

Itoh, N., T. Kouzai, and Y. Koide. 1994. Efficient transformation of *Pseudomonas* strains with pNI vectors by electroporation. *Biosci. Biotechnol. Biochem.* 58:1306-1308.

Iwasaki, K., H. Uchiyama, O. Yagi, T. Kurabayashi, K. Ishizuka, and Y. Takamura. 1994. Transformation of *Pseudomonas putida* by electroporation. *Biosci. Biotechnol. Biochem.* 58:851-854.

James, K., M.O.C. Motherway, F. Bottacini, and D. Van Sinderen. 2016. *Bifidobacterium breve* UCC2003 metabolises the human milk oligosaccharides lacto-N-tetraose and lacto-N-neo-tetraose through overlapping, yet distinct pathways. *Sci. Rep.* 6:38560.

Jensen, P.R., and K. Hammer. 1998. The sequence of spacers between the consensus sequences modulates the strength of

prokaryotic promoters. *Appl. Environ. Microbiol.* 64:82-87.

Jeong, D.-W., J.-H. Lee, K.H. Kim, and H.J. Lee. 2006. A food-grade expression/secretion vector for *Lactococcus lactis* that uses an α-galactosidase gene as a selection marker. *Food Microbiol.* 23:468-475.

Jia, W., H. Li, L. Zhao, and J.K. Nicholson. 2008. Gut microbiota: a potential new territory for drug targeting. *Nat. Rev. Drug Discov*. 7:123.

Jiang, T., A. Mustapha, and D.A. Savaiano. 1996. Improvement of lactose digestion in humans by ingestion of unfermented milk containing *Bifidobacterium longum*. *J. Dairy Sci.* 79:750-757.

Johnson, E.L., S.L. Heaver, W.A. Walters, and R.E. Ley. 2017. Microbiome and metabolic disease: revisiting the bacterial phylum Bacteroidetes. *J. Mol. Med.* 95:1-8.

Jones-Hall, Y.L., and C.H. Nakatsu. 2016. The Intersection of TNF, IBD and the Microbiome. *Gut microbes*. 7:58-62.

Joutsjoki, V., S. Luoma, M. Tamminen, M. Kilpi, E. Johansen, and A. Palva. 2002. Recombinant *Lactococcus* starters as a potential source of additional peptidolytic activity in cheese ripening. *J. Appl. Microbiol.* 92:1159-1166.

Juers, D.H., B.W. Matthews, and R.E. Huber. 2012. LacZ β-galactosidase: structure and function of an enzyme of historical and molecular biological importance. *Protein Sci.* 21:1792-1807.

Juers, D.H., T.D. Heightman, A. Vasella, J.D. McCarter, L. Mackenzie, S.G. Withers, and B.W. Matthews. 2001. A structural view of the action of *Escherichia coli* (lac Z) β-galactosidase. *Biochemistry*. 40:14781-14794.

Kadlec, R., and M. Jakubec. 2014. The effect of prebiotics on adherence of probiotics. *J. Dairy Sci.* 97:1983-1990.

Kamran, A., Z. Bibi, A. Aman, and S.A.U. Qader. 2016. Lactose hydrolysis approach: isolation and production of β-galactosidase from newly isolated *Bacillus* strain B-2. *Biocatal. Agric. Biotechnol*. 5:99-103.

Khosaka, T., M. Kiwaki, and B. Rak. 1983. Two site-specific endonucleases BinSI and BinSII from *Bifidobacterium infantis*. *FEBS Lett.* 163:170-174.

Khosaka, T., T. Sakurai, H. Takahashi, and H. Saito. 1982. A new site-specific endonuclease BbeI from *Bifidobacterium breve*: Restriction endonucleases;; 3-cohesive termini; DNA sequence analysis. *Gene*. 17:117-122.

Kim, H.S., and S.E. Gilliland. 1983. *Lactobacillus acidophilus* as a dietary adjunct for milk to aid lactose digestion in humans. *J. Dairy Sci.* 66:959-966.

Kim, IH., MS. Park, and GE. Ji. 2003. Characterization of adhesion of *Bifidobacterium* sp. BGN4 to human enterocyte-like Caco-2 cells. *J. Microbiol. Biotechnol*. 13:276-281.

Kim, JY., Y. Wang, MS. Park, and GE. Ji. 2010. Improvement of

transformation efficiency through in vitro methylation and SacII site mutation of plasmid vector in *Bifidobacterium longum* MG1. *J. Microbiol. Biotechnol.* 20:1022-1026.

Kim, N., and GE. Ji. 2006. Modulatory activity of *Bifidobacterium* sp. BGN4 cell fractions on immune cells. *J. Microbiol. Biotechnol.* 16:584.

Kim, N., J. Kunisawa, M.N. Kweon, G. Eog Ji, and H. Kiyono. 2007. Oral feeding of *Bifidobacterium bifidum* (BGN4) prevents CD4(+) CD45RB(high) T cell-mediated inflammatory bowel disease by inhibition of disordered T cell activation. *Clin. Immunol.* 123:30-39.

Kim, Y., K. Han, S. Oh, S. You, and S. Kim. 2005. Optimization of technical conditions for the transformation of *Lactobacillus acidophilus* strains by electroporation. *J. Appl. Microbiol.* 99:167-174.

Kleessen, B., G. Stoof, J. Proll, D. Schmiedl, J. Noack, and M. Blaut. 1997. Feeding resistant starch affects fecal and cecal microflora and short-chain fatty acids in rats. *J. Anim. Sci.* 75:2453-2462.

Konikoff, T., and U. Gophna. 2016. *Oscillospira*: a central, enigmatic component of the human gut microbiota. *Trends Microbiol.* 24:523-524.

Kopp-Hoolihan, L. 2001. Prophylactic and therapeutic uses of probiotics: a review. *J. Am. Diet. Assoc.* 101:229-241.

Koropatkin, N.M., E.A. Cameron, and E.C. Martens. 2012. How glycan metabolism shapes the human gut microbiota. *Nat. Rev. Microbiol.* 10:323.

Ku, S., H.J. You, and G.E. Ji. 2009. Enhancement of anti-tumorigenic polysaccharide production, adhesion, and branch formation of *Bifidobacterium bifidum* BGN4 by phytic acid. *Food Sci. Biotechnol.* 18:749-754.

Ku, S., M.S. Park, G.E. Ji, and H.J. You. 2016. Review on *Bifidobacterium bifidum* BGN4: Functionality and Nutraceutical Applications as a Probiotic Microorganism. *Int. J. Mol. Sci.* 17:1544-1566.

Kumar, A., and M. Bansal. 2018. Modulation of Gene Expression by Gene Architecture and Promoter Structure. *IntechOpen.* 76051.

Landete, J.M. 2017. A review of food-grade vectors in lactic acid bacteria: from the laboratory to their application. *Crit. Rev. Biotechnol.* 37:296-308.

Lee, M.J., Z. Zang, EY. Choi, H.K. Shin, and G.-E. Ji. 2002. Cytoskeleton reorganization and cytokine production of macrophages by bifidobacterial cells and cell-free extracts. *J. Microbiol. Biotechnol.* 12:398-405.

Lee, S., N. Koo, and S. Oh. 2006. Regulatory Effect on Specific IgE Response of *Bifidobacterium bifidum*(bgn4 Strain) in Murine Model of Peanut Allergy. *J. Allergy Clin. Immunol.* 117:S204.

Leroy, F., and L. De Vuyst. 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends Food Sci. Technol.* 15:67-78.

Levri, K.M., K. Ketvertis, M. Deramo, J.H. Merenstein, and F. D'amico. 2005. Do probiotics reduce adult lactose intolerance? A systematic review. *J. Fam. Pract.* 54:613-621.

Lewis, Z.T., G. Shani, C.F. Masarweh, M. Popovic, S.A. Frese, D.A. Sela, M.A. Underwood, and D.A. Mills. 2016. Validating bifidobacterial species and subspecies identity in commercial probiotic products. *Pediatr. Res.* 79:445.

Li, J., and Y. Zhang. 2014. Relationship between promoter sequence and its strength in gene expression. *Eur. Phys. J.* 37:86.

Li, J., W. Zhang, C. Wang, Q. Yu, R. Dai, and X. Pei. 2012. *Lactococcus lactis* expressing food-grade β-galactosidase alleviates lactose intolerance symptoms in post-weaning Balb/c mice. *Appl. Microbiol. Biotechnol.* 96:1499-1506.

Li, R., T.M. Takala, M. Qiao, H. Xu, and P.E. Saris. 2011. Nisin-selectable food-grade secretion vector for *Lactococcus lactis*. *Biotechnol. Lett.* 33:797-803.

Lin, MY., CL. Yen, and SH. Chen. 1998. Management of lactose maldigestion by consuming milk containing lactobacilli. *Dig. Dis. Sci.* 43:133-137.

Lin, MY., D. Savaiano, and S. Harlander. 1991. Influence of nonfermented dairy products containing bacterial starter

cultures on lactose maldigestion in humans. *J. Dairy Sci.* 74:87–95.

Lin, MY., J.A. Dipalma, M.C. Martini, C.J. Gross, S.K. Harlander, and D.A. Savaiano. 1993. Comparative effects of exogenous lactase (β–galactosidase) preparations on *in vivo* lactose digestion. *Dig. Dis. Sci.* 38:2022–2027.

Liu, C.Q., P. Su, N. Khunajakr, Y.M. Deng, S. Sumual, W. Kim, J. Tandianus, and N. Dunn. 2005. Development of food-grade cloning and expression vectors for *Lactococcus lactis*. *J. Appl. Microbiol.* 98:127–135.

Löfblom, J., N. Kronqvist, M. Uhlén, S. Ståhl, and H. Wernérus. 2007. Optimization of electroporation-mediated transformation: *Staphylococcus carnosus* as model organism. *J. Appl. Microbiol.* 102:736–747.

Lomer, M., G. Parkes, and J. Sanderson. 2008. lactose intolerance in clinical practice–myths and realities. *Aliment. Pharmacol. Ther.* 27:93–103.

Maksimainen, M., N. Hakulinen, J.M. Kallio, T. Timoharju, O. Turunen, and J. Rouvinen. 2011. Crystal structures of *Trichoderma reesei* β–galactosidase reveal conformational changes in the active site. *J. Struct. Biol.* 174:156–163.

Marsili, R., H. Ostapenko, R. Simmons, and D. Green. 1981. High performance liquid chromatographic determination of organic acids in dairy products. *J. Food Sci.* 46:52–57.

Marteau, P. 2009. Bacterial flora in inflammatory bowel disease. *Dig. Dis.* 27:99-103.

Mary, O., C. Motherway, D. Watson, F. Bottacini, T.A. Clark, R.J. Roberts, J. Korlach, P. Garault, C. Chervaux, and J.E. van Hylckama Vlieg. 2014. Identification of restriction-modification systems of *Bifidobacterium animalis* subsp. *lactis* CNCM I-2494 by SMRT sequencing and associated methylome analysis. *PloS one*. 9:e94875.

Masood, M.I., M.I. Qadir, J.H. Shirazi, and I.U. Khan. 2011. Beneficial effects of lactic acid bacteria on human beings. *Crit. Rev. Microbiol.* 37:91-98.

Matsuki, T., K. Watanabe, J. Fujimoto, Y. Kado, T. Takada, K. Matsumoto, and R. Tanaka. 2004a. Quantitative PCR with 16S rRNA gene-targeted species-specific primers for analysis of human intestinal bifidobacteria. *Appl. Environ. Microbiol.* 70:167-173.

Matsuki, T., K. Watanabe, J. Fujimoto, T. Takada, and R. Tanaka. 2004b. Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. *Appl. Environ. Microbiol.* 70:7220-7228.

McCracken, A., M.S. Turner, P. Giffard, L.M. Hafner, and P. Timms. 2000. Analysis of promoter sequences from *Lactobacillus* and *Lactococcus* and their activity in several *Lactobacillus* species. *Arch. Microbiol.* 173:383-389.

McDonough, F.E., A.D. Hitchins, N.P. Wong, P. Wells, and C. Bodwell. 1987. Modification of sweet acidophilus milk to improve utilization by lactose-intolerant persons. *Am. J. Clin. Nutr.* 45:570-574.

Medow, M.S., K.D. Thek, L.J. Newman, S. Berezin, M.S. Glassman, and S.M. Schwarz. 1990. β-galactosidase tablets in the treatment of lactose intolerance in pediatrics. *Am. J. Dis. Child.* 144:1261-1264.

Milani, C., A. Hevia, E. Foroni, S. Duranti, F. Turroni, G.A. Lugli, B. Sanchez, R. Martin, M. Gueimonde, and D. Van Sinderen. 2013. Assessing the fecal microbiota: an optimized ion torrent 16S rRNA gene-based analysis protocol. *PloS one*. 8:e68739.

Milani, C., G.A. Lugli, S. Duranti, F. Turroni, L. Mancabelli, C. Ferrario, M. Mangifesta, A. Hevia, A. Viappiani, and M. Scholz. 2015. Bifidobacteria exhibit social behavior through carbohydrate resource sharing in the gut. *Sci. Rep.* 5:15782.

Miller, J.F., W.J. Dower, and L.S. Tompkins. 1988. High-voltage electroporation of bacteria: genetic transformation of *Campylobacter jejuni* with plasmid DNA. *Proc. Natl. Acad. Sci.* 85:856-860.

Misselwitz, B., D. Pohl, H. Frühauf, M. Fried, S.R. Vavricka, and M. Fox. 2013. Lactose malabsorption and intolerance: pathogenesis, diagnosis and treatment. *United European gastroenterology J.* 1:151-159.

Mitsuoka, T. 1990. Bifidobacteria and their role in human health. *J. Ind. Microbiol*. 6:263–267.

Møller, P.L., F. Jørgensen, O.C. Hansen, S.M. Madsen, and P. Stougaard. 2001. Intra–and extracellular β–galactosidases from *Bifidobacterium bifidum* and *B. infantis*: molecular cloning, heterologous expression, and comparative characterization. *Appl. Environ. Microbiol.* 67:2276–2283.

Monsan, P., and F. Paul. 1995. Enzymatic synthesis of oligosaccharides. *FEMS Microbiol. Rev.* 16:187–192.

Montalto, M., V. Curigliano, L. Santoro, M. Vastola, G. Cammarota, R. Manna, A. Gasbarrini, and G. Gasbarrini. 2006. Management and treatment of lactose malabsorption. *World J. Gastroenterol.* 12:187.

Mustapha, A., T. Jiang, and D.A. Savaiano. 1997. Improvement of lactose digestion by humans following ingestion of unfermented acidophilus milk: influence of bile sensitivity, lactose transport, and acid tolerance of *Lactobacillus acidophilus*. *J. Dairy Sci.* 80:1537–1545.

Nagy, Z., T. Kiss, A. Szentirmai, and S. Biró. 2001. β–Galactosidase of *Penicillium chrysogenum*: production, purification, and characterization of the enzyme. *Protein Expression Purif.* 21:24–29.

Newcomer, A.D., H. Park, P.C. O'Brien, and D.B. McGill. 1983.

Response of patients with irritable bowel syndrome and lactase deficiency using unfermented acidophilus milk. *Am. J. Clin. Nutr*. 38:257−263.

Nguyen, T.−h., B. Splechtna, M. Steinböck, W. Kneifel, H.P. Lettner, K.D. Kulbe, and D. Haltrich. 2006. Purification and characterization of two novel β−galactosidases from *Lactobacillus reuteri*. *J. Agric. Food Chem.* 54:4989−4998.

Nguyen, TT., G. Mathiesen, L. Fredriksen, R. Kittl, TH. Nguyen, V.G. Eijsink, D. Haltrich, and C.K. Peterbauer. 2011. A food−grade system for inducible gene expression in *Lactobacillus plantarum* using an alanine racemase−encoding selection marker. *J. Agric. Food Chem.* 59:5617−5624.

Nguyen, T.−T., H.−M. Nguyen, B. Geiger, G. Mathiesen, V.G. Eijsink, C.K. Peterbauer, D. Haltrich, and T.−H. Nguyen. 2015. Heterologous expression of a recombinant lactobacillal β−galactosidase in *Lactobacillus plantarum*: effect of different parameters on the sakacin P−based expression system. *Microb. Cell Fact*. 14:30.

Nguyen, T.−T., H.A. Nguyen, S.L. Arreola, G. Mlynek, K. Djinovic´−Carugo, G. Mathiesen, T.−H. Nguyen, and D. Haltrich. 2012. Homodimeric β−galactosidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081: expression in *Lactobacillus plantarum* and biochemical characterization. *J. Agric. Food Chem.* 60:1713−1721.

Nivetha, A., and V. Mohanasrinivasan. 2017. Mini review on role of β-galactosidase in lactose intolerance. *Mater. Sci. Eng.* 263:022046.

O'Connell Motherway, M., J. O'driscoll, G.F. Fitzgerald, and D. Van Sinderen. 2009. Overcoming the restriction barrier to plasmid transformation and targeted mutagenesis in *Bifidobacterium breve* UCC2003. *Microb. Biotechnol.* 2:321-332.

O'Connell Motherway, M., M. Kinsella, G.F. Fitzgerald, and D. van Sinderen. 2013. Transcriptional and functional characterization of genetic elements involved in galacto-oligosaccharide utilization by *Bifidobacterium breve* UCC 2003. *Microb. Biotechnol.* 6:67-79.

O' Callaghan, A., F. Bottacini, M.C. Motherway, and D. Van Sinderen. 2015. Pangenome analysis of *Bifidobacterium longum* and site-directed mutagenesis through by-pass of restriction-modification systems. *BMC Genomics*. 16:832.

Oak, S.J., and R. Jha. 2018. The effects of probiotics in lactose intolerance: A systematic review. *Crit. Rev. Food Sci. Nutr.* 59(11):1675-1683.

Oh, S.Y., S.Y. Youn, M.S. Park, H.-G. Kim, N.-I. Baek, Z. Li, and G.E. Ji. 2017. Synthesis of β-galactooligosaccharide using bifidobacterial β-galactosidase purified from recombinant *Escherichia coli*. *J. Microbiol. Biotechnol.* 27:1392-1400.

Ohlsson, J.A., M. Johansson, H. Hansson, A. Abrahamson, L. Byberg,

A. Smedman, H. Lindmark-Månsson, and Å. Lundh. 2017. Lactose, glucose and galactose content in milk, fermented milk and lactose-free milk products. *Int. Dairy J.* 73:151-154.

Oliveira, P.H., D.M. Prazeres, and G.A. Monteiro. 2009. Deletion formation mutations in plasmid expression vectors are unfavored by runaway amplification conditions and differentially selected under kanamycin stress. *J. Biotechnol.* 143:231-238.

Onwulata, C.I., D.R. Rao, and P. Vankineni. 1989. Relative efficiency of yogurt, sweet acidophilus milk, hydrolyzed-lactose milk, and a commercial lactase tablet in alleviating lactose maldigestion. *Am. J. Clin. Nutr.* 49:1233-1237.

Palomino, M.M., C. Sanchez-Rivas, and S.M. Ruzal. 2009. High salt stress in *Bacillus subtilis*: involvement of PBP4* as a peptidoglycan hydrolase. *Res. Microbiol.* 160:117-124.

Palomino, M.M., M.C. Allievi, M. Prado-Acosta, C. Sanchez-Rivas, and S.M. Ruzal. 2010. New method for electroporation of *Lactobacillus* species grown in high salt. *J. Microbiol. Methods.* 83:164-167.

Park, MS., HW. Moon, and GE. Ji. 2003. Molecular characterization of plasmid from *Bifidobacterium longum*. *J. Microbiol. Biotechnol.* 13:457-462.

Park, M.J., J.H. Lee, K.A. Kim, J.S. Kim, H.C. Jung, I.S. Song, and C.Y. Kim. 1999. The changes in the breath hydrogen concentration

after the ingestion of *Bifidobacterium breve* KY-16 in the lactose malabsorbers. *Korean J. Gastroenterol.* 34:741-748.

Park, M.J., M.S. Park, and G.E. Ji. 2018. Improvement of electroporation-mediated transformation efficiency for a *Bifidobacterium* strain to a reproducibly high level. *J. Microbiol. Methods*.

Park, M.S., D.W. Shin, K.H. Lee, and G.E. Ji. 1999. Sequence analysis of plasmid pKJ50 from *Bifidobacterium longum*. *Microbiology*. 145:585-592.

Park, S.Y., G.E. Ji, Y.T. Ko, H.K. Jung, Z. Ustunol, and J.J. Pestka. 1999. Potentiation of hydrogen peroxide, nitric oxide, and cytokine production in RAW 264.7 macrophage cells exposed to human and commercial isolates of *Bifidobacterium*. *Int. J. Food Microbiol.* 46:231-241.

Parvez, S., K.A. Malik, S. Ah Kang, and H.Y. Kim. 2006. Probiotics and their fermented food products are beneficial for health. *J. Appl. Microbiol.* 100:1171-1185.

Pedersen, M.B., S.L. Iversen, K.I. Sørensen, and E. Johansen. 2005. The long and winding road from the research laboratory to industrial applications of lactic acid bacteria. *FEMS Microbiol. Rev.* 29:611-624.

Pyne, M.E., M. Moo-Young, D.A. Chung, and C.P. Chou. 2013. Development of an electrotransformation protocol for genetic manipulation of *Clostridium pasteurianum*. *Biotechnol. Biofuels*.

6:50-69.

Rabot, S., J. Rafter, G.T. Rijkers, B. Watzl, and J.-M. Antoine. 2010. Guidance for substantiating the evidence for beneficial effects of probiotics: impact of probiotics on digestive system metabolism. *J. Nutr*. 140:677S-689S.

Reuter, G. 2001. The *Lactobacillus* and *Bifidobacterium* microflora of the human intestine: composition and succession. *Curr. Issues Intestinal Microbiol.* 2:43-53.

Rivière, A., M. Selak, D. Lantin, F. Leroy, and L. De Vuyst. 2016. Bifidobacteria and butyrate-producing colon bacteria: importance and strategies for their stimulation in the human gut. *Front Microbiol*. 7:979.

Rooks, M.G., P. Veiga, L.H. Wardwell-Scott, T. Tickle, N. Segata, M. Michaud, C.A. Gallini, C. Beal, J.E. van Hylckama-Vlieg, and S.A. Ballal. 2014. Gut microbiome composition and function in experimental colitis during active disease and treatment-induced remission. *ISME J*. 8:1403.

Rosenberg, Z.M.-M. 2006. Current trends of β-galactosidase application in food technology. *J. Food Nutr. Res*. 45:47-54.

Rossi, M., P. Brigidi, A.G.V. y Rodriguez, and D. Matteuzzi. 1996. Characterization of the plasmid pMB1 from *Bifidobacterium longum* and its use for shuttle vector construction. *Res. Microbiol.* 147:133-143.

Roy, D., J.-L. Berger, and G. Reuter. 1994. Characterization of

dairy-related *Bifidobacterium* spp. based on their β-galactosidase electrophoretic patterns. *Int. J. Food Microbiol.* 23:55-70.

Russell, W.R., L. Hoyles, H.J. Flint, and M.-E. Dumas. 2013. Colonic bacterial metabolites and human health. *Curr. Opin. Microbiol.* 16:246-254.

Sadhu, A., I. Ghosh, Y. Moriyasu, A. Mukherjee, and M. Bandyopadhyay. 2018. Role of cerium oxide nanoparticle-induced autophagy as a safeguard to exogenous H_2O_2-mediated DNA damage in tobacco BY-2 cells. *Mutagenesis*. 33:161-177.

Saier Jr, M.H., and T.M. Ramseier. 1996. The catabolite repressor/activator (Cra) protein of enteric bacteria. *J. Bacteriol.* 178:3411.

Saltzman, J.R., R.M. Russell, B. Golner, S. Barakat, G.E. Dallal, and B.R. Goldin. 1999. A randomized trial of *Lactobacillus acidophilus* BG2FO4 to treat lactose intolerance. *Am. J. Clin. Nutr*. 69:140-146.

Sánchez, B., M. Fernández-García, A. Margolles, G. Clara, and P. Ruas-Madiedo. 2010. Technological and probiotic selection criteria of a bile-adapted *Bifidobacterium animalis* subsp. *lactis* strain. *Int. Dairy J.* 20:800-805.

Sanders, M.E. 2008. Probiotics: definition, sources, selection, and uses. *Clin. Infect. Dis.* 46:S58-S61.

Sangrador-Vegas, A., C. Stanton, D. Van Sinderen, G. Fitzgerald, and R. Ross. 2007. Characterization of plasmid pASV479 from *Bifidobacterium pseudolongum* subsp. *globosum* and its use for expression vector construction. *Plasmid*. 58:140-147.

Sani, R., S. Chakraborti, R. Sobti, P. Patnaik, and U. Banerjee. 1999. Characterization and some reaction-engineering aspects of thermostable extracellular β-galactosidase from a new *Bacillus* species. *Folia Microbiol.* 44:367.

Saqib, S., A. Akram, S.A. Halim, and R. Tassaduq. 2017. Sources of β-galactosidase and its applications in food industry. *Biotech*. 7:79.

Savaiano, D. 2011. Lactose intolerance: an unnecessary risk for low bone density. *Nestle Nutr. Workshop Ser. Pediatr. Program.* 67: 161-171.

Savaiano, D.A., A. AbouElAnouar, D.E. Smith, and M.D. Levitt. 1984. Lactose malabsorption from yogurt, pasteurized yogurt, sweet acidophilus milk, and cultured milk in lactase-deficient individuals. *Am. J. Clin. Nutr*. 40:1219-1223.

Serafini, F., F. Turroni, S. Guglielmetti, L. Gioiosa, E. Foroni, V. Sanghez, A. Bartolomucci, M.O.C. Motherway, P. Palanza, and D. van Sinderen. 2012. An efficient and reproducible method for transformation of genetically recalcitrant bifidobacteria. *FEMS Microbiol. Lett.* 333:146-152.

Sharma, A.D., J. Singh, and P.K. Gill. 2007. Ethanol mediated

enhancement in bacterial transformation. *Electron. J. Biotechnol.* 10:166-168.

Shigekawa, K., and W.J. Dower. 1988. Electroporation of eukaryo and prokaryotes: a general approach to the introduction of macromolecules into cells. *Biotechniques*. 6:742-751.

Shimamura, S., F. Abe, N. Ishibashi, H. Miyakawa, T. Yaeshima, T. Araya, and M. Tomita. 1992. Relationship between oxygen sensitivity and oxygen metabolism of *Bifidobacterium* species. *J. Dairy Sci.* 75:3296-3306.

Shkoporov, A.N., B.A. Efimov, E.V. Khokhlova, J.L. Steele, L.I. Kafarskaia, and V.V. Smeianov. 2008. Characterization of plasmids from human infant *Bifidobacterium* strains: sequence analysis and construction of *E. coli-Bifidobacterium* shuttle vectors. *Plasmid*. 60:136-148.

Si, JM., YC. Yu, YJ. Fan, and SJ. Chen. 2004. Intestinal microecology and quality of life in irritable bowel syndrome patients. *World J. Gastroenterol.* 10:1802.

Silva, F., J.A. Queiroz, and F.C. Domingues. 2012. Evaluating metabolic stress and plasmid stability in plasmid DNA production by *Escherichia coli*. *Biotechnol. Adv.* 30:691-708.

Sinha, A.K. 1972. Colorimetric assay of catalase. *Anal. Biochem.* 47:389-394.

Sivan, A., L. Corrales, N. Hubert, J.B. Williams, K. Aquino-Michaels, Z.M. Earley, F.W. Benyamin, Y.M. Lei, B. Jabri, and M.-L.

Alegre. 2015. Commensal *Bifidobacterium* promotes antitumor immunity and facilitates anti-PD-L1 efficacy. *Science.* 350(6264): 1084-1089

Smart, J.B., C.J. Pillidge, and J.H. Garman. 1993. Growth of lactic acid bacteria and bifidobacteria on lactose and lactose-related mono-, di-and trisaccharides and correlation with distribution of β-galactosidase and phospho-β-galactosidase. *J. Dairy Res.* 60:557-568.

Splechtna, B., T.H. Nguyen, R. Zehetner, H.P. Lettner, W. Lorenz, and D. Haltrich. 2007. Process development for the production of prebiotic galacto-oligosaccharides from lactose using β-galactosidase from *Lactobacillus* sp. *Biotechnol. J.* 2:480-485.

Steidler, L., S. Neirynck, N. Huyghebaert, V. Snoeck, A. Vermeire, B. Goddeeris, E. Cox, J.P. Remon, and E. Remaut. 2003. Biological containment of genetically modified *Lactococcus lactis* for intestinal delivery of human interleukin 10. *Nat. Biotechnol.* 21:785.

Stiles, M.E., and W.H. Holzapfel. 1997. Lactic acid bacteria of foods and their current taxonomy. *Int. J. Food Microbiol.* 36:1-29.

Storz, G., and J.A. Imlayt. 1999. Oxidative stress. *Curr. Opin. Microbiol.* 2:188-194.

Suau, A., R. Bonnet, M. Sutren, J.-J. Godon, G.R. Gibson, M.D. Collins, and J. Doré. 1999. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular

species within the human gut. *Appl. Environ. Microbiol.* 65:4799-4807.

Suzuki, H. 2012. Host-mimicking strategies in DNA methylation for improved bacterial transformation. *IntechOpen.* 51691.

Thannickal, V.J., and B.L. Fanburg. 2000. Reactive oxygen species in cell signaling. *Am. J. Physiol.* 279:L1005-L1028.

Thompson, K., and M.A. Collins. 1996. Improvement in electroporation efficiency for *Lactobacillus plantarum* by the inclusion of high concentrations of glycine in the growth medium. *J. Microbiol. Methods*. 26:73-79.

Torres, D.P., M.d.P.F. Gonçalves, J.A. Teixeira, and L.R. Rodrigues. 2010. Galacto-oligosaccharides: Production, properties, applications, and significance as prebiotics. *Compr. Rev. Food Sci. Food Saf*. 9:438-454.

Trevors, J.T., B.M. Chassy, W.J. Dower, and H.P. Blaschek. 1991. Electrotransformation of bacteria by plasmid DNA. *Food Sci. Hum. Nutr*. 265-290.

Troise, A.D., E. Bandini, R. De Donno, G. Meijer, M. Trezzi, and V. Fogliano. 2016. The quality of low lactose milk is affected by the side proteolytic activity of the lactase used in the production process. *Food Res. Int.* 89:514-525.

Turroni, F., C. Milani, S. Duranti, C. Ferrario, G.A. Lugli, L. Mancabelli, D. van Sinderen, and M. Ventura. 2018. Bifidobacteria and the infant gut: an example of co-evolution and natural selection.

Cell. Mol. Life Sci. 75:103-118.

Turroni, F., C. Peano, D.A. Pass, E. Foroni, M. Severgnini, M.J. Claesson, C. Kerr, J. Hourihane, D. Murray, and F. Fuligni. 2012. Diversity of bifidobacteria within the infant gut microbiota. *PloS one*. 7:e36957.

Turroni, F., S. Duranti, F. Bottacini, S. Guglielmetti, D. Van Sinderen, and M. Ventura. 2014. *Bifidobacterium bifidum* as an example of a specialized human gut commensal. *Front Microbiol*. 5:437.

Ullmann, A. 1996. Catabolite repression: a story without end. *Res. Microbiol.* 147:455-458.

Van Laere, K.M., T. Abee, H.A. Schols, G. Beldman, and A.G. Voragen. 2000. Characterization of a Novel β-Galactosidase from *Bifidobacterium adolescentis* DSM 20083 Active towards Trans-galactooligosaccharides. *Appl. Environ. Microbiol.* 66:1379-1384.

Ventura, M., S. O'flaherty, M.J. Claesson, F. Turroni, T.R. Klaenhammer, D. Van Sinderen, and P.W. O'toole. 2009. Genome-scale analyses of health-promoting bacteria: probiogenomics. *Nat. Rev. Microbiol*. 7:61.

Vijaranakul, U., M.J. Nadakavukaren, B. De Jonge, B.J. Wilkinson, and R.K. Jayaswal. 1995. Increased cell size and shortened peptidoglycan interpeptide bridge of NaCl-stressed *Staphylococcus aureus* and their reversal by glycine betaine. *J. Bacteriol.* 177:5116-5121.

Vonk, R.J., G.A. Reckman, H.J. Harmsen, and M.G. Priebe. 2012. Probiotics and lactose intolerance. *IntechOpen.* 51424.

Walters, W.A., Z. Xu, and R. Knight. 2014. Meta-analyses of human gut microbes associated with obesity and IBD. *FEBS Lett.* 588:4223-4233.

Wang, Y., J.Y. Kim, M.S. Park, and G.E. Ji. 2012. Novel *Bifidobacterium* promoters selected through microarray analysis lead to constitutive high-level gene expression. *J. Microbiol.* 50:638-643.

Weber, F.J., and J.A. de Bont. 1996. Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. *Biochim. Biophys. Acta. Biomembranes*. 1286:225-245.

Wegmann, U., J. Klein, I. Drumm, O. Kuipers, and B. Henrich. 1999. Introduction of peptidase genes from *Lactobacillus delbrueckii* subsp. *lactis* into *Lactococcus lactis* and controlled expression. *Appl. Environ. Microbiol.* 65:4729-4733.

Wei, M.Q., K.A. Ellem, P. Dunn, M.J. West, C.X. Bai, and B. Vogelstein. 2007. Facultative or obligate anaerobic bacteria have the potential for multimodality therapy of solid tumours. *Eur. J. Cancer*. 43:490-496.

Wirth, R., A. Friesenegger, and S. Fiedler. 1989. Transformation of various species of gram-negative bacteria belonging to 11 different genera by electroporation. *Mol. Gen. Genet.*

216:175-177.

Wright, E.K., M.A. Kamm, S.M. Teo, M. Inouye, J. Wagner, and C.D. Kirkwood. 2015. Recent advances in characterizing the gastrointestinal microbiome in Crohn's disease: a systematic review. *Inflamm. Bowel Dis.* 21:1219-1228.

Wu, Z., L. Li, W. Ma, Y. Yu, and Y. Chen. 2000. A new index for microbial colonization resistance of the bowel-the ratio of B/E. *Zhejiang Yu Fang Yi Xue*. 7:4-5.

Xenos, K., S. Kyroudis, A. Anagnostidis, and P. Papastathopoulos. 1998. Treatment of lactose intolerance with exogenous beta-D-galactosidase in pellet form. *Eur. J. Drug Metab. Pharmacokinet.* 23:350-355.

You, H.J., D.K. Oh, and G.E. Ji. 2004. Anticancerogenic effect of a novel chiroinositol-containing polysaccharide from *Bifidobacterium bifidum* BGN4. *FEMS Microbiol. Lett.* 240:131-136.

Youn, S.Y., M.S. Park, and G.E. Ji. 2012. Identification of the β-glucosidase gene from *Bifidobacterium animalis* subsp. *lactis* and its expression in *B. bifidum* BGN4. *J. Microbiol. Biotechnol.* 22:1714-1723.

Yuan, T., P. Yang, Y. Wang, K. Meng, H. Luo, W. Zhang, N. Wu, Y. Fan, and B. Yao. 2008. Heterologous expression of a gene encoding a thermostable β-galactosidase from *Alicyclobacillus acidocaldarius*. *Biotechnol. Lett.* 30:343-348.

Zhang, W., C. Wang, C. Huang, Q. Yu, H. Liu, C. Zhang, and X. Pei. 2011. Construction and expression of food-grade β-galactosidase gene in *Lactococcus lactis*. *Curr. Microbiol.* 62:639-644.

Zuo, F., R. Yu, X. Feng, G.B. Khaskheli, L. Chen, H. Ma, and S. Chen. 2014. Combination of heterogeneous catalase and superoxide dismutase protects *Bifidobacterium longum* strain NCC2705 from oxidative stress. *Appl. Microbiol. Biotechnol.* 98:7523-7534.

국문 초록

본 연구는 분자 유전학적 기법을 통해 *Bifidobacterium* 유래 β-galactosidase를 고발현하는 재조합 bifidobacteria를 개발하여 이를 *in vitro* lactose 가수분해 및 *in vivo* lactose intolerance (LI) 동물연구에 활용해 *Bifidobacterium* 유래 β-galactosidase의 활용범위를 넓히는 것을 목적으로 하였다. 또한 부수적으로, 이러한 유전자 재조합 bifidobacteria가 장차 인체에 적용될 수 있도록 현재까지 구축되지 않은 bifidobacteria용 food-grade vector를 위한 selection marker를 개발하고자 하였다.

β-galactosidase 고발현 bifidobacteria의 개발에 앞서, bifidobacteria에 β-galactosidase gene을 효과적으로 도입하기 위해 bifidobacteria의 낮은 electroporation 매개 transformation 효율을 재현성있게 높은 수준으로 개선하기 위한 사전 연구가 수행되었다. 실험 결과, cell wall weakening agent (NaCl)와 cell membrane permeabilizing agent (Ethanol)를 적용하고 electrical parameter를 2.5 kV에서 3 kV로 조정하였을 때 본 연구의 transformation host로 사용된 *B. bifidum* BGN4의 transformation 효율이 10^3에서 10^5 CFU / μg DNA로 72배 높아졌다. 최적화된 방법은 다른 *Bifidobacterium* species에도 적용할 수 있었다.

이제 본 연구로 돌아가 *Bifidobacterium* 유래 β-galactosidase gene을 이용하여 β-galactosidase 고발현 bifidobacteria를 개발하기

위해, 가장 높은 수준의 β-galactosidase 활성을 갖는 *Bifidobacterium* strain 중 하나인 *B. longum* RD47의 β-galactosidase gene을 여러 bifidobacteria 유래 promoter들과 조합하여 BGN4에서 발현시켰다. 개발된 재조합 bifidobacteria 중 BGN4-G1은 BGN4보다 2.5 - 4.2 배 높고, RD47보다 4.3 - 9.6 배 높은 β-galactosidase 활성을 나타냈다. 또한 자연에 존재하는 35개 유산균들과 비교하였을 때에도 BGN4-G1의 효소 활성이 월등히 뛰어났다. 시판우유와 BGN4-G1을 반응시켰을 때, BGN4-G1은 발효되면서 기존 발효유의 약 2배에 달하는 lactose 제거율을 보였다. 시판우유와 BGN4-G1의 crude enzyme extract를 반응시켰을 때에는 BGN4-G1의 β-galactosidase가 2시간 동안 51%의 유당을 가수분해하였다. 이는 50 ㎍의 단백질을 함유하는 crude enzyme extract를 첨가하였을 때의 결과였기 때문에 crude enzyme extract의 양을 증가시킬 경우 더 큰 효과가 기대된다. 이들 결과들로 미루어 보아, his-tag을 붙여 BGN4-G1으로부터 β-galactosidase를 정제하면 이를 lactose-free milk나 fermented milk 생산에 활용할 수 있을 것으로 사료된다.

Lactose intolerance (LI)는 소화관에서 β-galactosidase가 결여되거나 부족하여 발생하는 질병으로, 세계 성인 인구의 약 75%가 겪고 있기 때문에 공중보건관리 측면에서 전세계적인 문제로 간주된다. 최근, *Bifidobacterium* species에 속하는 균주가 LI의 잠재적인 치료제로서 주목받고 있다. 그러나 *Bifidobacterium*이 LI에 효과가 있는지 없는지에 대하여 이전 연구들에서 서로 상반된 결과들이 도출되어왔다. 우리는 이러한 상반된 결과의 원인이 실험에 사용된 균주

간의 β-galactosidase 활성 차이에 있다는 가설을 세웠고, 이를 입증하기 위해 *in vivo* 실험을 수행하기로 하였다. 이에 따라 β-galactosidase 저발현 bifidobacteria, wild-type bifidobacteria, β-galactosidase 고발현 bifidobacteria를 LI model mouse로 알려진 이유기 이후의 Balb/c mouse에 각각 투여함으로써 bifidobacteria의 β-galactosidase 활성이 LI 완화에 미치는 영향을 확인해보았다. 이전 실험에서 개발되었던 BGN4-G1을 β-galactosidase 고발현 bifidobacteria로, BGN4를 wild-type bifidobacteria로 사용하였으며, BGN4보다 β-galactosidase 활성이 약 3.2배 낮은 BGN4 돌연변이체인 BGN4-bgr을 개발하여 이를 β-galactosidase 저발현 bifidobacteria로 사용하였다.

Balb/c 마우스를 7개의 실험군으로 그룹화하였는데 각 군은 다음과 같다. 1) wild-type BGN4 투여군, 2) BGN4-pBES2 투여군, 3) BGN4-bgr 투여군, 4) BGN4-G1 투여군, 5) LI만 유도된 untreated군, 6) LI 유도 직전에 lactase가 투여된 lactase-treated군, 7) LI가 유도되지 않은 uninduced군. 모든 mouse에 생균 용액이나 생리식염수를 2주간 경구투여 하였으며 그 후, lactose를 투여해 6시간동안 LI 증상을 관찰하였다. 실험 결과, BGN4-G1 투여군은 대변 빈도와 대변의 무게가 untreated군에 비해 현저히 감소하였고 장 운동성이 억제되었다. BGN4-G1 투여군의 LI 완화 효과는 양성대조군으로 사용된 lactase-treated군보다 더 컸으며 심지어 uninduced군의 수준에까지 도달하였다. 한편, BGN4 및 BGN4-bgr 투여군의 대변 빈도와 대변의 무게는 untreated군에 비해 감소하는 경향이 있었지만 유의한 차이는 발견되지 않았다. 장 운동성의 경우,

BGN4-bgr 투여군과 달리 BGN4 투여군에서 untreated군에 비해 장 운동성이 유의적으로 억제된 결과가 나타났다. 이는 우리의 가설대로 bifidobacteria의 β-galactosidase 활성이 높아짐에 따라 LI 완화정도가 증가함을 시사한다.

이전의 연구들에서 LI는 장내 미생물군집과도 연관성을 지닌다고 보고되어왔다. 우리는 bifidobacteria의 β-galactosidase 활성에 따른 LI 완화 기전을 이러한 관점에서 조사해보고자 BGN4-bgr, BGN4, BGN4-G1 또는 생리식염수를 2주간 투여한 mouse의 대변에서 장내 미생물 분석을 실시하였다. 또한 SCFA와 lactate는 장내 미생물의 발효산물이기 때문에 이들 mouse의 맹장 내 SCFA와 lactate의 함량을 부수적으로 측정해보았다. 실험 결과, 다음과 같은 몇 가지 의미있는 결과들이 도출되었다. 1) 장 건강과 관련된 몇몇 미생물들의 비율이 bifidobacteria 투여군과 untreated군 사이에서 구분되는 경향이 있었다. 2) BGN4-G1군에서의 *Bifidobacterium* 비율이 BGN4 및 BGN4-bgr군에 비해 증가하는 경향이 나타났다. 3) SCFA와 lactate의 balance는 bifidobacteria 투여군과 untreated군 사이에서 구분되었다. 4) SCFA와 lactate의 변화 양상은 BGN4-bgr군을 제외한 BGN4-G1 및 BGN4군에서 비슷한 패턴을 보였다.

BGN4-G1이 LI 증상을 유의적으로 완화하였기 때문에, food-grade vector를 통해 G1을 발현하는 BGN4-G1이 개발되면 LI 완화 균주로서 활용될 가능성이 매우 높을 것으로 기대된다. 그러나 현재까지 bifidobacteria용 food-grade vector가 개발되지 못해 재조합 bifidobacteria들이 인체에 적용될 수 없는 실정이다. 이에 따라, 본

연구에서는 bifidobacteria용 food-grade vector 개발을 위한 selection marker로서 SOD와 catalase gene의 이용 가능성을 평가해보았다. 먼저, lactic acid bacteria 유래의 SOD 및 catalase gene을 BGN4 내에서 발현시켰으며 해당 recombinant를 BGN4-SK라 명명하였다. 그 후, BGN4-SK에서 catalase가 가장 높은 수준으로 발현될 수 있는 최적 조건과 wild-type BGN4와 BGN4-SK의 생존율이 명확하게 구분될 수 있는 H_2O_2 stress 조건을 탐색해보았다. 이를 적용하여 10^8 CFU의 wild-type BGN4 내에서 10^2 CFU의 BGN4-SK를 선별하는 실험을 수행해보았는데, 설정된 H_2O_2 stress 하에서 대부분의 균이 죽어 오직 34개의 콜로니만이 살아남았으며 그 중 2개의 콜로니가 sequencing 결과 BGN4-SK로 확인되었다. 이는 H_2O_2 stress에 의한 selection pressure가 대다수의 wild-type BGN4 내에서 극소수의 recombinant BGN4를 선별하는 데에 적합함을 시사한다. 본 실험은 bifidobacteria용 food-grade vector 개발을 진전시킬 수 있는 효과적인 selection marker 후보군을 찾았다는 데에 의미를 지닌다.

주요어 : 베타 갈락토시다아제, 재조합 비피도박테리아, 비피도박테리움 롱검 알디47, 비피도박테리움 비피덤 비지엔포, 전기천공 매개 형질전환 효율, 유당 가수분해, 유당불내증, 푸드그레이드 벡터

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